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INVESTIGATION OF THE ACUTE INFLAMMATORY RESPONSE IN CROHN'S DISEASE

By

Daniel Joseph Benjamin Marks

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
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Abstract

Most theories concerning the primary cause of Crohn's disease focus on over-activation of the immune response. Paradoxically, the defect may instead relate to diminished acute inflammation. Neutrophil accumulation to sites of dermal trauma has been shown to be reduced. Were the same phenomenon to occur in the gut, it might impair bacterial clearance thus provoking granuloma formation.

In this thesis, a novel technique demonstrated attenuated neutrophil accumulation following trauma to the bowel. A modified skin window technique linked this failure of migration to defective IL-8 production. Polymorphisms in *CARD15*, associated with susceptibility to Crohn's disease, compounded the problem by abrogating the normal pro-inflammatory action of the protein but did not underlie the basic phenotype. Consequently, the response of macrophages to other inflammatory agonists was examined. IL-8 production was also impaired in Crohn's disease after stimulation with wound fluid, C5a or TNF-α.

The response of Crohn's patients to gut bacteria was assessed directly *in vivo* by subcutaneous injection of killed *Escherichia coli*. This elicited substantial local inflammation in controls, manifested by an NO-mediated increase in blood flow. The response was considerably lower in Crohn's patients, particular those with colonic disease. In contrast, acute phase reactants were highest in the latter, supporting the hypothesis that an impaired local response can drive a systemic pro-inflammatory state.

The demonstration of attenuated acute inflammation in Crohn's disease may have important implications for understanding its pathogenesis and targeting novel therapies.

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Abbreviations

AIDS, acquired immunodeficiency syndrome; ALP, alkaline phosphatase; AS, ankylosing spondylitis; 5-ASA, 5-aminosalicylate; ANOVA, analysis of variance

BSA, bovine serum albumin

CARD, caspase-recruitment domain; CD, Crohn's disease; CGD, Chronic Granulomatous Disease; cGMP, cyclic guanosine monophosphate; CH, compound heterozygous; COX, cyclooxygenase; CRP, C-reactive protein

DEPC, diethylpyrocarbonate; DPX, distrene/plasticizer/xylene; DSS, dextran sodium sulfate; DTPA, diethylenetriaminopentaacetic acid; DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; EN, erythema nodosum; ESR, erythrocyte sedimentation rate

FAE, follicle-associated epithelium; FAP, familial adenomatous polyposis; FKHR, Forkhead family transcription factor

GADPH, glyceraldehyde-3-phosphate dehydrogenase; GALT, gut-associated lymphoid tissue; G(M)-CSF, granulocyte (macrophage)-colony stimulating factor; GVB, gelatin veronal buffer

HBSS, Hanks Balanced Salt solution; HLA, human leukocyte antigen; HRP, horseradish peroxidase; HZ, homozygous

IFN, interferon; Ig, immunoglobulin; IL, interleukin

LBP, lipopolysaccharide-binding protein; l-NMMA, NG-monomethyl-L-arginine acetate; LOX, lipooxygenase; LPS, lipopolysaccharide; LRR, leucine-rich repeat, LTB, leukotriene B

MALDI-TOF, matrix-assisted laser desorption time-of-flight; MAP, *Mycobacterium avium paratuberculosis*; MDP, muramyl dipeptide; MHC, major histocompatibility complex; MPO, myeloperoxidase; MW, molecular weight

NADPH, nicotinamide dinucleotide phosphate; NBD, nucleotide-binding domain; NF-κB, nuclear factor-κB; NO(S), nitric oxide (synthase); NSAID, non-steroidal anti-inflammatory drug; NSB, non-specific binding

PAGE, polyacrylamide gel electrophoresis; PAPA, pyogenic sterile arthritis, pyoderma gangrenosum and acne; PBS, phosphate-buffered saline; (RT)-PCR, (reverse transcriptase)-polymerase chain reaction; PDE, phosphodiesterase; PEG, polyethylene glycol; PG, pyoderma gangrenosum; PGE, prostaglandin E; PKC, protein kinase C; PPACK, H-d-Phe-Pro-Arg-CH_2Cl; PSC, primary sclerosing cholangitis

RA, rheumatoid arthritis; RICK, RIP-like interacting CLARP kinase; ROS, reactive oxygen species

SAA, serum amyloid A; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; SH, simple heterozygous; SNP, single nucleotide polymorphism

TA, total activity; TACE, TNF-α-converting enzyme; TBS(T), Tris-buffered saline (Tween-20); TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinase; TLR, Toll-like receptor; TMB, tetramethylbenzidine; TNF-α, tumour necrosis factor-α

UC, ulcerative colitis; UCLH, University College London Hospital

WCC, white cell count; WT, wild type

Chapter 1: Introduction

1.1 Clinical features of Crohn's disease

1.1.1 Crohn's disease manifests as a granulomatous enterocolitis

Crohn's disease is a chronic inflammatory granulomatous disorder primarily affecting the gastrointestinal tract and associated with considerable lifelong morbidity. The first description has been ascribed to Giovanni Battista Morgagni (1682-1771), who reported a 20-year-old man suffering from protracted diarrhoea and colicky abdominal pain and who later died from a perforated ileum[1]. Subsequent patients were described in Sweden by John Berg in 1898 and in Warsaw by Antoni Lesniowski in 1903[2]. In 1913, Dalziel published the first case series of nine patients with disseminated thickening of the terminal ileum, some of whom also had colonic involvement[3]. The disease reached prominence in 1932, when Crohn and colleagues published their case series of 14 patients with regional ileitis[4].

The incidence of Crohn's disease rose substantially during the latter part of the twentieth century[5] for reasons that remain poorly understood, although this has now become relatively constant. The prevalence varies geographically, with rates highest in northern Europe and North America, approaching 100/100,000 people[6]. It affects females slightly more frequently than males. Although it can develop at any time of life, onset typically occurs between the ages of 20-29, with a second smaller peak between 60-79 years of age. Of the various environmental risk factors examined to date, cigarette smoking remains the strongest, conferring a five-fold increase in susceptibility and a higher incidence of exacerbations[7].

Patients present with inflammation that can occur at any point throughout the alimentary canal. The majority of lesions develop in the terminal ileum (approximately 40%), colon (30%) or both (30%), with relative sparing of the upper gastrointestinal tract and rectum[8]. These manifest symptomatically with abdominal pain, diarrhoea, intestinal bleeding and malnutrition, resulting in considerable morbidity. Other organs can also be affected, producing the extraintestinal features associated with this disease. These include inflammation in the eyes (uveitis and episcleritis), skin (erythema nodosum and pyoderma gangrenosum), joints (peripheral arthritis, sacroiliitis and ankylosing spondylitis), peri-anal region (abscesses and fistulae) and the hepatobiliary system (sclerosing cholangitis). The natural history of Crohn's disease typically follows a relapsing-remitting course, with intermittent flares disrupting periods of inactivity. Mortality appears only slightly greater than that of the general population, with a reduction in life expectancy of approximately five years[9]. This small excess seems to relate to the increased requirements for surgery and the modestly raised (approximately five-fold[10]) risk of colorectal cancer.

At endoscopy, the earliest visible features include erythema and oedema of the intestinal mucosa, with the formation of multiple aphthous ulcers (Fig. 1.1A) that can coalesce into confluent serpiginous longitudinal tracks[11] (Fig. 1.1B). Inflammation often occurs in multiple discrete, well-demarcated patches separated by areas of normal mucosa, producing skip lesions and a cobblestone appearance (Fig. 1.1C). Prolonged active disease can lead to the development of strictures through extensive fibrosis (Fig. 1.1D) and fistula formation by extension of inflammation through the serosa.

Microscopic features of biopsy specimens recovered from areas of affected mucosa include a transmural inflammatory infiltrate (Fig. 1.2A,B); occasional fibrosis or fissuring (Fig. 1.2C); lymphoid hyperplasia and frequently granuloma formation[11] (Fig. 1.2D); and distortion of crypt architecture in longstanding disease (Fig. 1.2E). A granuloma comprises a collection of macrophages with a surrounding mantle of lymphocytes. These typically form in response to the presence of non-digestible foreign material[12]. In some diseases, such as tuberculosis, the offending antigen is known. In other conditions, the provoking stimulus remains unidentified: these include sarcoidosis, Wegener's granulomatosis and Crohn's disease. Although most reports in the literature suggest that granulomata develop in only 50% of patients[13], this may be an underestimate. These numbers are based on clinical studies with tissue obtained for diagnostic purposes, in which usually no more than twelve biopsies are taken from the small and large bowel combined; this is probably greatly underpowered. A study in which resected intestines from Crohn's patients were step-sectioned showed that granulomata were present throughout the tract, including in areas of otherwise apparently normal mucosa[14]. The potential for a type I error due to sampling is therefore high, due to both obtaining tissue from the wrong location or at the wrong time, since granulomata are dynamic structures[15].

In active disease, therefore, histology reveals substantial inflammation. Both mononuclear and polymorphonuclear leukocytes infiltrate the mucosa (Fig. 1.2E), driven by local production of inflammatory mediators. Inflamed bowel shows increased production of many pro-inflammatory cytokines, including

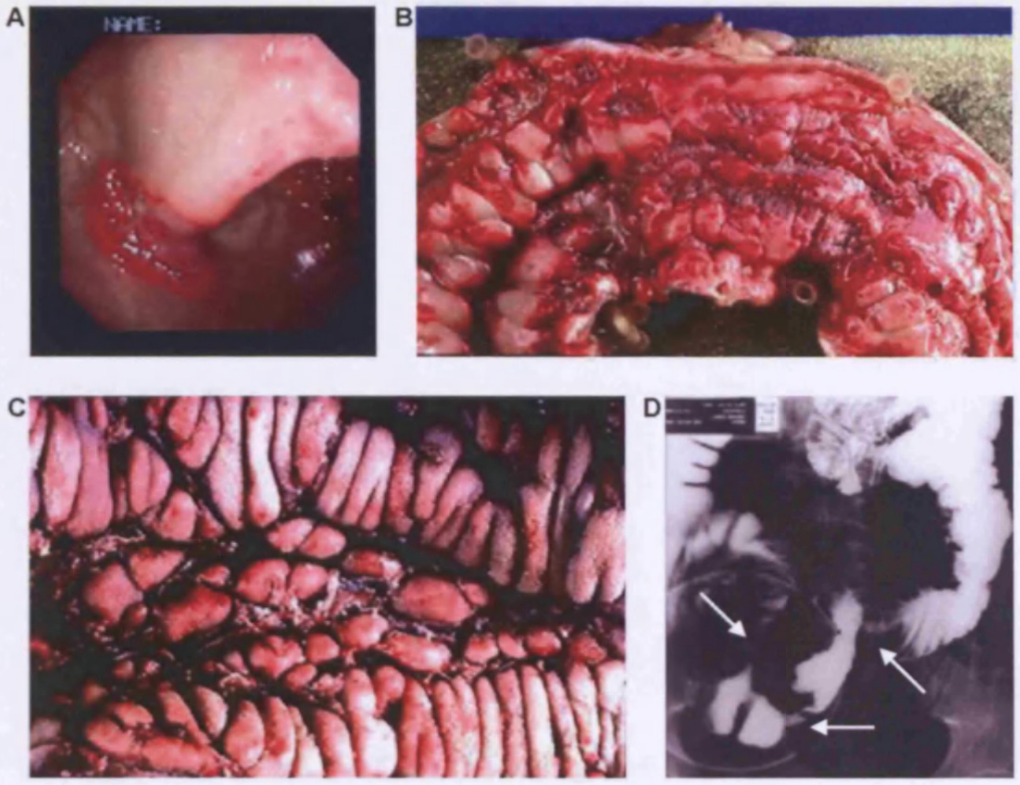


Figure 1.1 Typical macroscopic appearances of Crohn's lesions. **(A)** Ulcer in the colon. **(B)** Linear ulcers. **(C)** Cobblestone mucosa. **(D)** Barium follow-through showing separation of bowel wall loops (indicating bowel wall oedema) and stricturing (arrows).

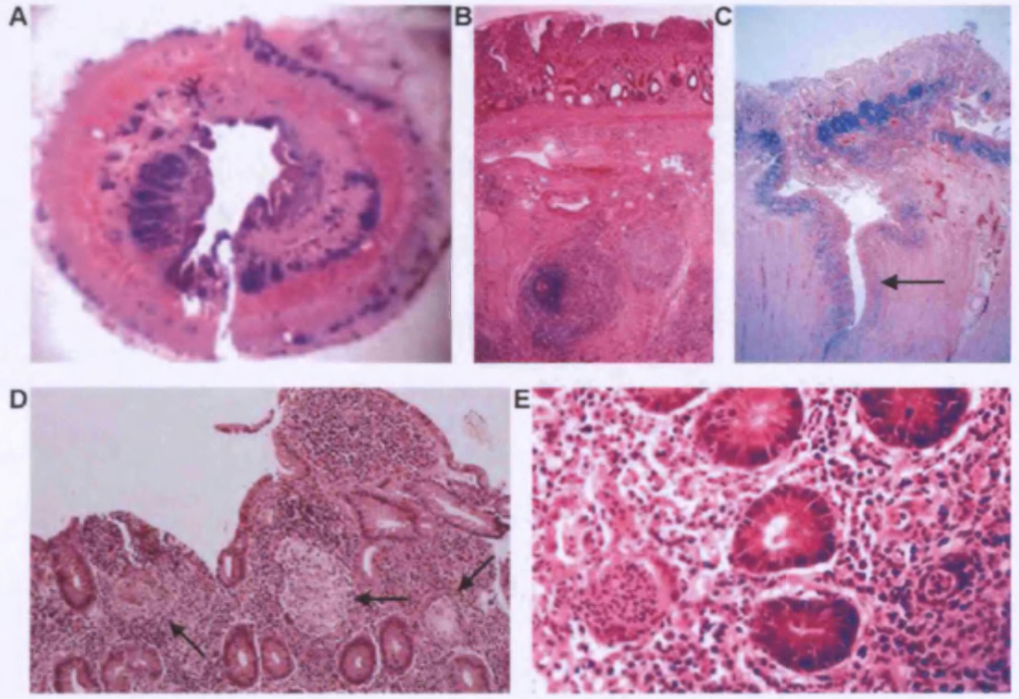


Figure 1.2 Typical microscopic appearances of Crohn's lesions. **(A,B)** Transmural inflammation. **(C)** Fissure formation (arrow). **(D)** Granulomata (arrows). **(E)** Cryptitis with a mixed leukocytic infiltrate.

interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α)[16]. More specifically, the cytokine pattern corresponds to a Th1 immunological reaction, with raised concentrations of interleukin-2 (IL-2), interleukin-12 (IL-12) and interferon-γ (IFN-γ)[17].

1.1.2 Immunosuppression provides the mainstay of treatment

Whilst management of Crohn's disease requires a multidisciplinary approach, drugs that suppress the immune system play a central role. Corticosteroids remain the most effective pharmacological agents for active disease[18]. These bind to intracellular receptors leading to a number of potentially beneficial effects, including reduced leukocyte activation and survival; decreased activation of the typically pro-inflammatory transcription factor nuclear factor-κB (NF-κB); and attenuated induction of pro-inflammatory mediators including cytokines and eicosanoids[19].

Unfortunately corticosteroids carry a heavy burden of side effects, particularly with chronic use. In view both of this and the paucity of evidence for their efficacy in the maintenance of remission[20], alternative agents have been developed for long-term management. The 5-aminosalicylate (5-ASA) derivatives, such as sulfasalazine (Fig. 1.3A) and mesalazine (Fig. 1.3B), appear moderately effective for active disease, although the evidence for benefit in relapse prevention remains equivocal[21]. Chemical modifications allow preferential release in the small or large bowel. Whilst less potent, they possess a similar spectrum of anti-inflammatory actions to corticosteroids.

Azathioprine (Fig. 1.3C) and its metabolite 6-mercaptopurine (Fig. 1.3D) are used principally in disease otherwise dependent on or refractory to

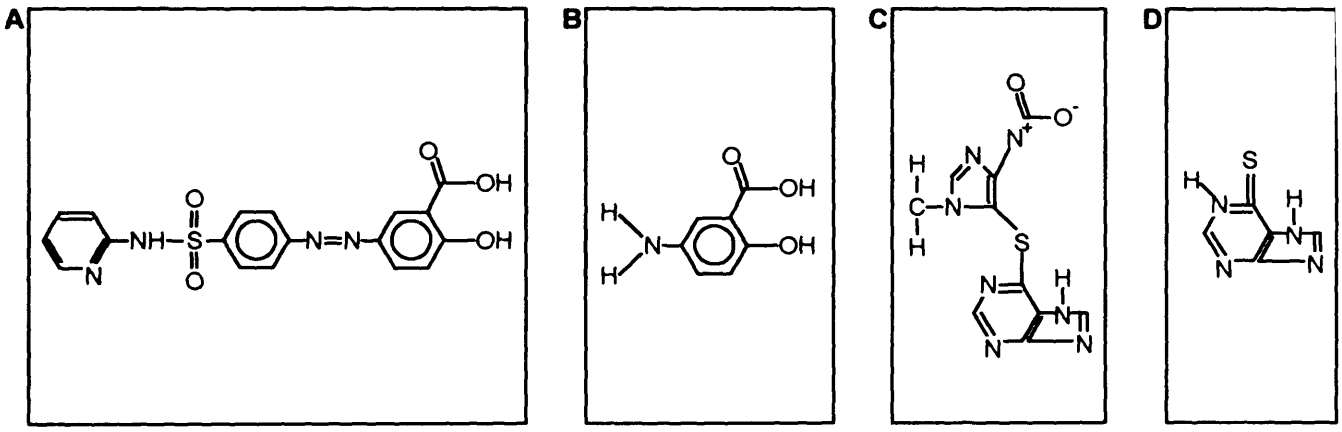


Figure 1.3 Chemical structures of therapeutic drugs. **(A)** sulfasalazine, **(B)** mesalazine, **(C)** azathioprine and **(D)** 6-mercaptopurine.

corticosteroids[22]. The proposed mechanism of action relates to their ability as purine analogues to inhibit DNA synthesis and thus lymphocyte proliferation, although modulation of neutrophil function has also been documented. Other immunosuppressive agents, including methotrexate and cyclosporine, are employed far less frequently.

Antibodies that recognize TNF-α comprise a recent addition to the pharmacological repertoire for Crohn's disease. These were initially investigated under the hypothesis that pro-inflammatory TNF-α plays a central role in driving chronic inflammation, and that its neutralization might prove therapeutically beneficial[23]. Clinical trials with a chimeric antibody (Infliximab) and more recently a fully humanized antibody (Adalimumab) have demonstrated efficacy, particularly in patients with fistulating or otherwise refractory disease[24]. Their mechanism of action, however, does not appear straightforward. TNF-α blockade was originally pioneered for the treatment of chronic inflammation in rheumatoid arthritis. Two classes of drug were developed: antibodies against TNF-α and recombinant TNF-α receptors (Etanercept and Onercept). Both bind TNF-α with similar efficacy and both ameliorate inflammation in rheumatoid arthritis[25]. In contrast, the receptors have proven ineffective for Crohn's disease[24,26], suggesting that simple blockade of TNF-α is insufficient in this condition. The true mechanism of action appears to relate to the ability of the antibodies to bind and cross-link membrane-bound TNF-α on the surface of lymphocytes, initiating apoptosis as well as complement-mediated and antibody-dependent cytotoxicity[23].

Whilst effective at suppressing inflammation, none of these therapies are curative. All have side effects, and none guarantee prevention of relapse. The

same is true of surgical removal of affected bowel: around 50% of patients develop symptomatic recurrence and more than 90% exhibit endoscopic features of active disease at the anastomosis within 1 year of operation[27]. Such procedures are thus usually reserved to treat secondary complications of Crohn's disease: typical indications include disease refractory to medical management; the presence of persistent intestinal obstruction or fistulae; or emergency complications such as bowel perforation. Despite its temporary efficacy, however, approximately 60% of patients require surgery at some point in their lives[28].

1.1.3 Alternative inflammatory enteritides

Crohn's disease comprises only one of the inflammatory enteritides. Ulcerative colitis remains the most significant other idiopathic inflammatory bowel disease. Although it can produce similar symptoms, the pathology differs with restriction of inflammation to the large bowel (although a backwash ileitis can occur), in continuous segments usually starting at the rectum without intervening unaffected mucosa[29]. Leukocytes infiltrate only superficially into the epithelium, accompanied by marked goblet cell depletion but not granuloma formation. The immunological pattern varies from Crohn's disease: the process appears Th2-driven with high levels of interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13)[30]. The study of ulcerative colitis as a control inflammatory disorder is crucial, in that it allows distinction of factors related specifically to Crohn's disease compared to those secondary to any chronic inflammation affecting the gastrointestinal tract.

Other, rarer idiopathic inflammatory bowel diseases include lymphocytic, eosinophilic, microscopic and collagenous colitis, the latter often associated with use of non-steroidal anti-inflammatory drugs (NSAID)[31]. These exhibit distinct pathological features and almost certainly represent discrete disease entities. In approximately 15% of patients, pathological investigations reveal only non-specific inflammatory changes consistent with more than one of these enteritides or another cause such as infection[32]. This indeterminate colitis has been interpreted by some to indicate a spectrum of disease with Crohn's disease and ulcerative colitis at either end but an underlying unifying pathogenesis. A failure to arrive at a definitive diagnosis may, however, simply reflect sampling error in obtaining pathological specimens from the bowel.

1.2 Mucosal immunology

1.2.1 The aetiology of Crohn's disease remains unclear

The exuberant inflammatory reaction in the gastrointestinal tract underlies the generation of symptoms and morbidity in Crohn's disease. The driving force behind this inflammation remains unknown. Most research has focused on the immunological characteristics of established lesions and drawn inferences about their initiation. The aetiological factors responsible for disease onset and relapse could be very different from those underlying persistence.

A major difficulty in defining the cause of Crohn's disease stems from its heterogeneity amongst patients, as evidenced by the different sites of bowel involvement, disease behaviours and associated clinical features. It is probable that multiple genetic and environmental factors, which differ between

individuals, give rise to a common syndrome. Such mechanistic differences may contribute to the well-established variations in clinical phenotype.

A wealth of experimental data indicates that Crohn's disease results from an abnormal interaction between the intestinal luminal contents, the mucosal surface and submucosal immune system[16]. The identity of the primary pathogenic factors remains highly contentious: some studies implicate abnormal infectious or particulate material within the bowel lumen, others a dysregulated innate or adaptive immune response. Compromise theories propose that a combination of these factors may ultimately prove important, with altered luminal contents acting on a susceptible immunological background. The relationship between the gastrointestinal flora and the mucosal immune system is explored below, together with the aberrations documented in Crohn's disease that may elicit bowel inflammation.

1.2.2 Commensal enteric flora

Microorganisms heavily colonize the adult bowel, with the numbers of bacteria constituting the normal gastrointestinal flora estimated as 10-fold greater than the number of other cells in the human body[33]. More than 500 different species have been reported to date, although recent genetic analyses of their complement of 16S ribosomal RNA genes in the distal intestine suggest that many more have yet to be cultured[34].

The neonate intestine remains sterile until shortly after birth, when it acquires a microbial flora within hours. In adults, the concentration of bacteria increases, with greatest numbers distally. The stomach and duodenum possess approximately 10^4 organisms/ml, predominantly *Lactobacilli*[33]. In the distal

ileum, the concentration rises to 10^6 organisms/ml, with the introduction of *Streptococci*. On reaching the caecum, numbers increase to 10^{12} organisms/gram of faecal material. This is largely due to the introduction of a major anaerobic component including *Bacteroides*, *Clostridia* and *Enterobacteriaceae*, but also a significant aerobic population of which *Escherichia coli* forms a principal constituent. These organisms are not normally pathogenic. Instead they participate in a symbiotic relationship with the host, playing important roles in the establishment and maintenance of mucosal structure and host defence, and the metabolism of certain vitamins and bile salts. The importance of the faecal flora in the pathogenesis of Crohn's disease is discussed later (see *1.3*).

1.2.3 The epithelial barrier

The gastrointestinal tract constitutes the major portal of entry of exogenous material into the body. The mucosal immune system, of which the epithelial barrier forms an important component, requires precise regulation so as to allow uptake of beneficial nutrients, not to overreact to the commensal bowel flora, and to eliminate potentially pathogenic microorganisms. Intestinal epithelial cells generally form a relatively impermeable physical barrier to the luminal contents, facilitated by tight junctions that impede paracellular transport[35]. Its integrity remains critical for preventing infiltration by luminal contents and subsequent inflammation, as illustrated by the spontaneous development of inflammatory bowel disease in transgenic mice expressing a dominant-negative mutant of the junctional adhesion protein cadherin[36].

In the small intestine, enterocytes project microvilli from their apical surface with a filamentous brush border; this anatomy further impedes

penetration by luminal contents[37]. Epithelial cells can also express proteins that recognize bacteria, including Toll-like receptors[38], and present antigens to mucosal leukocytes (see *1.2.5*), such as MHC-II molecules[39,40]. Additionally, they can participate in the orchestration of the inflammatory response by producing cytokines such as IL-8[41]. In order not to overreact to the normal intestinal flora, many of the pathogen receptors exhibit low or absent expression under basal conditions, with up-regulation when required. These include the peptidoglycan-responsive Toll-like receptor-2 (TLR2) and the LPS receptor complex of CD14, Toll-like receptor-4 (TLR4) and MD-2[42].

The epithelium also secretes a variety of compounds with antimicrobial actions into the bowel lumen. These derive from two principal cell types: goblet cells[43], which produce mucins and trefoil proteins, and Paneth cells[44]. Intestinal mucus forms a gel layer over the epithelium, acting as a semi-permeable barrier; its properties depend on the mucin content. Mucins are extremely large (up to 20 x 10^3 kDa), filamentous polypeptides that are heavily glycosylated (60-80% by mass), sulphated and sialated. This confers a highly negative surface charge and hydrophobicity[45,46]. They can be functionally subdivided into secretory (gel-forming), membrane-bound and soluble forms. As well as physically impeding microbial translocation, they also mimic pathogen adhesion sites[47,48] and store IgA, enhancing mucosal protection[49]. Trefoils constitute a group of small, cysteine-rich peptides expressed in a site-specific fashion in the intestine, with trefoil factor-3 production in the small and large intestines[50]. These both cross-link mucins to stabilise the mucus barrier[51], and stimulate epithelial restitution following injury[52].

The integrity of the mucus barrier appears vital for limiting bacterial ingress. In rat ileum in which this layer had been depleted, *in vitro* bacterial passage rose from 33.3% in controls to 100% in treated small bowel despite an intact epithelium[53]. Mice lacking mucin *Muc2*[45] or trefoil factor-3[54] proved extremely susceptible to dextran sodium sulfate-induced (DSS) colitis (see *1.8.1*); the phenotype in the latter was reversible on exogenous supplementation of this factor.

Paneth cells are generally restricted to the base of the crypts of Lieberkühn in the small intestine, although under inflammatory conditions they can arise in the oesophagus, stomach, pancreas and colon[55]. They synthesize a number of antibacterial molecules, including lysozyme, DNAse and α-defensin (cryptdin)[56]. The defensins are small, 3-4 kDa cationic peptides, produced by Paneth and myeloid cells[57]. Their antimicrobial action derives from their insertion into bacterial membranes to form pores of approximately 20 Å diameter; these dissipate electrochemical gradients leading to cell death[58]. Paneth cells release the contents of their secretory vesicles into the crypt lumen in response to a number of stimuli; these include whole bacteria, LPS, lipoteichoic acid, lipid A and muramyl dipeptide (*see 1.6.3*)[59].

The epithelium does not constitute an entirely continuous barrier to the luminal contents: food proteins can normally be detected in plasma[60] and a few gut bacteria within mesenteric lymph nodes[61]. Specialized M cells interrupt the enterocyte layer at regular intervals[62]. Their apical surface is thrown into microfolds, interspersed with clathrin-coated microdomains that mediate endocytosis of luminal particles[62,63]. Larger particles can also be engulfed by extension of cellular processes. These cells therefore mediate the transepithelial

transport of microbes, macromolecules and other foreign material. A pocket in their basolateral membrane protrudes against the apical surface, creating a docking site for subepithelial lymphocytes[64]. This assembly constitutes the follicle-associated epithelium (FAE), designed for the continuous sampling and surveillance of luminal contents by mucosal leukocytes (Fig. 1.4). The gut epithelium therefore merely limits but does not exclude luminal material from entering the tissues.

1.2.4 IgA

Numerous mononuclear leukocytes reside within the intestinal mucosa: principally macrophages, dendritic cells, lymphocytes and plasma cells. The latter secrete IgA, accounting for approximately 70% of all immunoglobulin production in the body[65]. Dendritic cells induce class switching to IgA production by B cells[66]. The latter then mature and re-circulate through the lymphatic system and blood stream[67], returning to the mucosa where they secrete IgA into the bowel lumen[68,69].

Whilst IgA plays an important role in neutralizing many enteric viruses[70], its antibacterial function remains less well defined. It coats the majority of commensal bacteria[71], limiting their non-specific ingress across the epithelial barrier[72,73] and blocking the action of potentially noxious microbial products[74,75]. It also enhances their recognition and uptake by mucosal leukocytes.

Passive immunization with purified IgA can protect against mucosal bacterial infection[76]. Mice deficient in the polymeric immunoglobulin receptor, which transports both IgA and IgM across the enterocyte layer, develop an enteropathy secondary to damage to the paracellular permeability barrier[77]. In

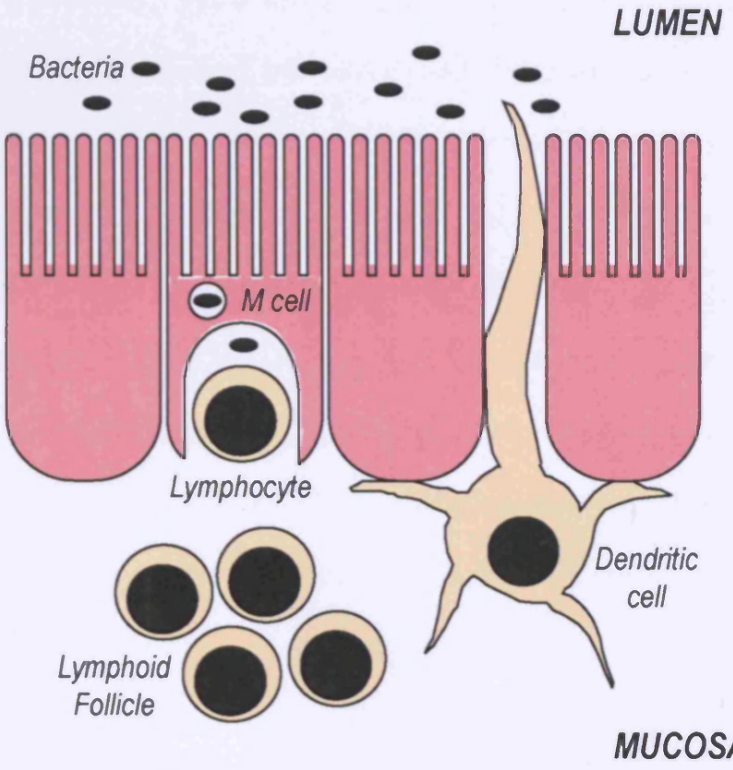


Figure 1.4 Follicle-associated epithelium. Specialized M cells endocytose luminal bacteria and pass them to lymphocytes sitting in a pocket in their basolateral membrane; these then migrate to lymphoid follicles. Dendritic cells reside superficially in the mucosa and extend processes through the epithelial cell barrier that continuously sample the luminal contents.

activation-induced cytidine deaminase knockout mice, which lack IgA but also other class-switched isotypes and affinity-matured IgM[78], luminal densities of commensal organisms rise. Antibody-deficient germ-free mice also exhibit a more persistent leak of these organisms through the mucosa on introduction of an intestinal flora[73,79]. These strains, however, subsequently adapt to terminate bacterial translocation[73], developing IgG recognizing the commensal flora[80].

IgA deficiency remains the commonest humoral immunodeficiency in humans, affecting up to 1 in 500-2,000 people[81]. Most develop no illness[82], perhaps partly related to compensatory up-regulation of IgM and IgG[81], although they exhibit some variation in their bowel flora constituents[83]. Rare cases of Crohn's disease arising in IgA-deficient individuals have been reported[84-86], but given the prevalence of both disorders this may be coincidental. These studies therefore demonstrate that IgA contributes to mucosal protection but is not essential.

1.2.5 Mucosal leukocytes and the gut-associated lymphoid tissue

In non-inflamed bowel, macrophages and dendritic cells probably constitute the first leukocytes to interact directly with opsonized microorganisms. Macrophages can express a number of pathogen recognition receptors that allow detection of bacteria. As with epithelial cells, many of these show minimal expression under resting conditions[87] but are up-regulated following bacterial invasion. Dendritic cells occupy a privileged position in which they extend processes through the epithelium[88] (Fig. 1.4). It has been suggested that this permits sampling and constant surveillance of soluble proteins and antigens within the lumen[89], and of the commensal flora, although direct evidence remains lacking. On detecting

potentially pathogenic material, macrophages and dendritic cells instigate acute inflammation by releasing cytokines that recruit and activate phagocytes from the surrounding microcirculation[90], and present antigen to lymphocytes initiating the adaptive immune response.

The gut-associated lymphoid tissue (GALT) consists of mucosal lymphocytes that tend to cluster. These aggregates are most prevalent in the small intestine where, in conjunction with other antigen-presenting cells, they form the Peyer's patches (numbering approximately 200 in adults)[91]. A further 10^5 smaller follicles line the remainder of the small intestine and colon[92]. Following antigen presentation, lymphocytes undergo clonal proliferation and differentiation. Naïve lymphocytes migrate to regional mesenteric lymph nodes where they undergo affinity maturation and, in the case of B cells, antibody class switching. Subsequently, lymphocytes that were activated in the GALT have a propensity to home back to the lamina propria, where they participate in the inflammatory response[93]. Helper $CD4^+$ T cells (Th) continue to present antigen and secrete cytokines. These augment the recruitment and response of other leukocytes, including cytotoxic $CD8^+$ T cells (Tc) that can directly eliminate infected host cells, and B cells and plasma cells. The latter cells both present antigen and secrete antibodies to optimize the recognition and removal of foreign material. A third population of $CD4^+$ suppressor T cells (Th3, T_{reg}) exert a negative, regulatory influence by secreting anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β)[94]. These limit the extent of inflammation and encourage resolution and healing (see *1.5.6*).

1.2.6 Commensal flora support mucosal immunity

The relationship between the normal gut flora and host immune system is closely regulated, since the former does not usually trigger a substantial inflammatory response. The anergy of mucosal leukocytes arises partly from intrinsic down-regulation of pathogen recognition receptors[87], but can also be induced by the microorganisms themselves. For example, many bacteria secrete proteins that interact with epithelial cells and leukocytes to inhibit NF-κB and consequently cytokine release[95].

Far from exerting a detrimental influence, commensal organisms play a vital role in establishing optimal mucosal immunity. Mice raised in germ-free environments possess very small Peyer's patches, and in human neonates the latter contain only poorly developed primary B cell follicles with few lymphocytes or plasma cells[96]. Furthermore, recognition of the normal flora through Toll-like receptors actually limits the extent of inflammation induced in the DSS model of colitis (see *1.8.1*)[97].

Gut bacteria often breach the epithelial barrier: they can routinely be detected in dendritic cells, within the Peyer's patches and local lymph nodes[98]. In contrast, they rarely spread past the mesenteric lymph nodes into the systemic circulation, due to prior removal by phagocytosis (see *1.5.2*). As discussed below (see *1.3.1*), the breakdown of some aspect of this homeostasis is believed to underlie the generation of Crohn's lesions.

1.3 Potential infective causes of Crohn's disease

1.3.1 Mucosal inflammation in Crohn's disease requires the luminal contents

Strong evidence supports a critical role for luminal constituents as environmental determinants of Crohn's disease. Inflammation most frequently occurs in the ileocaecal region and colon, both of which possess extremely high bacterial loads[33]. Furthermore, germ-free animals appear highly resistant to enteric inflammation[99], and possess minimal gut-associated lymphoid tissue or soluble IgA (see *1.2.4* and *1.2.5*)[96].

An elegant series of experiments demonstrate the requirement for the luminal contents in the generation of Crohn's lesions. In patients who have undergone an intestinal resection with stoma formation, inflammation does not arise in the distal de-functioned bowel[100,101]. In one study[102], 5 Crohn's patients had an ileal resection with an ileocolonic anastomosis; a proximal diverting ileostomy was then constructed excluding all distal bowel from intestinal transit. Six months later, biopsies were taken from the ileocolon then full transit restored. Six months after re-anastomosis, further biopsies were taken. None of the 5 patients demonstrated any inflammation whilst the faecal stream remained diverted; however, all developed endoscopic and histological recurrence following its reintroduction. In a control group of 75 patients with one-step surgery without diversion, 53 showed endoscopic recurrence in the neo-terminal ileum within six months of surgery.

Relapse can also be induced by experimental introduction of effluent from the ileostomy[103]. In three patients who had undergone an ileocolonic resection and anastomosis with temporary proximal ileostomy, intestinal fluids were instilled into the distal bowel for 8 days. Prior to this insult, the mucosa was

macroscopically and microscopically normal. Introduction of the faecal material induced focal infiltration of mononuclear cells, eosinophils and neutrophils into the mucosa, and up-regulation of molecular cell markers reflective of leukocyte activation and epithelioid macrophage transformation. The effects of the ileostomy effluent could be abrogated by prior passage through a 0.22 μm filter[104], which removes large particles and bacteria.

A number of effective treatments for Crohn's disease reduce the numbers of enteric bacteria and their fermentation products, as well as other dietary antigens. Metronidazole, an antibiotic with activity against anaerobes, has proven efficacious particularly in patients with peri-anal lesions[105]. Elemental diets consisting of amino acid feeds also reduce bacterial load. Several clinical trials demonstrate that these ameliorate disease activity, in some series as effectively as corticosteroids[106-108]. Surgical bypass of affected bowel is a recognized treatment, though rarely employed[109]. Modification of the bowel flora may also exert therapeutic effects as illustrated by the use of probiotics (living organisms that exert beneficial effects beyond basic nutrition)[110].

1.3.2 The enteric flora differs in Crohn's disease

Many have proposed a specific infectious origin for Crohn's disease, although unequivocal proof of transmissibility in humans has yet to be provided. The best evidence derives from inoculation of homogenates of affected Crohn's bowel into mice, which elicit a similar granulomatous reaction[111]; this can be prevented by pre-treatment of the tissues by autoclaving, storage at -20°C or irradiation[112]. This reaction is consistent with the substantial rate of Kveim test positivity reported in Crohn's disease[113].

The most prevalent bacteria at the sites of predilection for lesions have been detailed above (see *1.2.2*). Differential representation of commensal species has been reported in Crohn's disease[114], and perhaps more remain undiscovered due to limitations in culture techniques. In active disease, the numbers of *Escherichia coli* (of which an adherent-invasive subspecies has attracted recent interest[115]), *Bacteroides vulgatus*, *Mycoplasma pneumoniae* and L-phase bacteria appear increased, and those of *Bifidobacteria* and *Lactobacilli* reduced[116-119]. Two organisms have received particular attention as potential causal pathogens: *Mycobacterium avium paratuberculosis* (see *1.3.3*) and measles virus (see *1.3.4*).

Changes in commensal populations could, however, occur as a consequence of disease and not relate to the primary pathogenesis. Increased bowel transit due to diarrhoea and the use of medications with antibacterial properties could modify the resident flora. Furthermore, the distribution of bacteria within the bowel lumen may not reflect the population adjacent to the mucosa. Although anaerobes outnumber aerobes in the colonic faecal matter by 1,000-fold, they exist in equal proportions at the epithelial barrier[120]. Given their proximity to the mucosal immune system, these adherent bacteria are likely to prove most important for the generation of Crohn's lesions; greater numbers of these have been reported in inflammatory bowel disease patients[120].

1.3.3 Mycobacterium avium paratuberculosis

The suggestion that *Mycobacterium avium paratuberculosis* (MAP) infection underlies Crohn's disease accompanied the first case series of the syndrome[3]. In ruminants it causes Johne's disease, a granulomatous ileocolitis that many regard as the animal equivalent of Crohn's disease[121]. MAP persists within meat and

milk from these animals, providing a potential route of transmission. Although conventional histochemical techniques fail to reveal mycobacteria in Crohn's tissues, specialized culture of these samples can grow cell-wall deficient organisms termed spheroplasts. These later develop Ziehl-Neelsen-positive cell walls and contain a MAP genome[122]. Inoculation of animals with these isolates elicits variable phenotypes. Mice generate hepatic and splenic granulomata, many other species exhibit no detectable response[123] but, most impressively, goats develop a granulomatous ileitis following oral inoculation[124].

Case-control studies have examined the disease specificity of MAP, principally by comparing detection rates of its *IS900* DNA insertion element or serum antibodies in patients and controls. The results proved highly variable, not least due to methodological differences between studies[125]. Those that detected MAP DNA in Crohn's patients frequently reported similar rates in ulcerative colitis controls, and a significant incidence in healthy individuals[126-130]. Organisms isolated from Crohn's patients, however, exhibit greater viability in long-term culture[131].

Conclusive evidence for MAP pathogenicity in humans remains lacking. A frequently cited case report describes a 7-year-old boy who presented with granulomatous cervical lymphadenopathy, positive for MAP DNA, who 5 years later developed terminal ileitis consistent with Crohn's disease[132]. Whilst this story appears compelling, it warrants a proviso that the histological finding of caseating granulomata in the lymph node implicates *Mycobacterium scrofulaceum* (untested) as the causative pathogen. Subsequent inflammatory bowel disease could have arisen coincidentally, or the patient may have had an underlying immunodeficiency syndrome (see *1.5.3*).

Under the MAP hypothesis, anti-mycobacterial treatment should represent a cure. Early trials showed no efficacy[133-135], but these may have administered inappropriate agents or continued for an insufficient duration. More recent trials have included macrolide antibiotics but whilst these have reported some success[136-138], they were conducted as uncontrolled, open label studies. As such, they do not allow strong conclusions concerning treatment efficacy compared to spontaneous remission and placebo effects, or the long-term sustainability of benefits; nor did they comment on changes in the presence and viability of MAP.

A number of concerns have arisen concerning MAP as a candidate organism. Many have criticized Johne's disease as a model[139]. Affected animals develop neither the intestinal strictures or fistulae nor the extra-intestinal manifestations frequently observed in Crohn's disease. MAP can be directly visualized in Johne's disease tissue[140]. No documented evidence of zoonotic transmission exists[125], nor do the geographical distributions of the two diseases correspond[141]. Alterations in immunological background in Crohn's disease also argue against its pathogenicity. In patients treated with Infliximab, an inhibitor of TNF-α, or those who develop AIDS, the risk of reactivation of *Mycobacterium tuberculosis* and other atypical mycobacteria rises substantially; conversely bowel inflammation often remits[142,143].

Although MAP may persist abnormally in Crohn's disease, the case for causality remains weak. Any hypothesis invoking this as the primary aetiology needs to explain why carriage in healthy individuals does not lead to disease, accepting that *Mycobacterium tuberculosis* induces pathology only in a minority of those infected. This requires postulation of a susceptible immunological

background. Additionally, the failure to detect MAP in every patient indicates alternative explanations in these individuals.

1.3.4 Measles virus

The measles hypothesis of Crohn's disease arose following morphological studies that suggested a central role for a vasculitic process associated with paramyxoma-like particles, cellular inclusion bodies, and the detection of measles virus within lesions[144,145]. Subsequent epidemiological studies appeared, claiming increased susceptibility to Crohn's disease in individuals born within three months of measles epidemics[146], with maternal infection during pregnancy[147], and with measles vaccination[148].

Unfortunately, many of these results do not withstand close scrutiny. The morphological abnormalities actually closely resemble normal cellular structures[149]. Several attempts to replicate the detection of measles have failed[150-153], and the original methodology proven inadequate[154]. The epidemiological work contains severe ascertainment and selection biases that critically undermine its interpretation[155]. More rigorously designed studies refute any association[156-161], and the hypothesis is now discredited[155].

In conclusion, there is little evidence to support a primary pathogenic role for any single microorganism. The origin of Crohn's disease is unlikely to be fundamentally infectious. The more probable explanation remains an underlying abnormality in host defence on which the normal gut flora acts.

1.4 The adaptive immune system

1.4.1 Immune tolerance to normal gut contents

Lymphocytes usually react on encountering antigens recognized by their receptors, either membrane-bound immunoglobulin for B cells or T cell receptors for T cells. Tolerance is the mechanism by which the immune system prevents pathologic autoreactivity against self proteins[162]. The first stage occurs centrally within the thymus, which contains cells that express a variety of self antigens found in the periphery. T lymphocytes displaying too great an affinity for these epitopes are negatively selected and eliminated through apoptosis; the exception are a population of $CD4^+CD25^+$ T_{reg} cells[163].

Despite the efficacy of thymic selection, a number of potentially autoreactive effector cells escape into the systemic circulation. These are usually then restrained by a number of peripheral tolerogenic mechanisms[164]. These include homeostatic regulation by cytokines; T cell clonal anergy or apoptotic deletion in the absence of co-stimulatory signals from antigen-presenting cells; diversion of effector T cell phenotype towards an immature state (Th0); and suppression by T_{reg} cells through the release of IL-10 and TGF-β. Oral tolerance is a further phenomenon of unique immunological importance, in which oral exposure to antigenic peptides or proteins induces immunological anergy on subsequent re-challenge[165]. The means through which this occurs depends on the dose of antigen administered: low doses lead to active regulation by other cells, high doses to clonal deletion[166]. The mechanisms employed are similar to those of endogenous peripheral tolerance.

There have been claims that tolerance is broken in Crohn's disease. T cells isolated from the mucosa of healthy individuals react only against

heterologous bowel flora from different individuals; those from Crohn's patients appear to react to autologous flora[167]. Others have reported enhanced T cell proliferation following exposure to *Staphylococcus aureus* or LPS[168] and T cell resistance to apoptosis, associated with an increased ratio of Bcl-2:Bax[169]. These differences may, however, arise as a consequence of chronic inflammation, since the majority of cells were isolated from sites of active disease.

1.4.2 The case for autoimmunity

Many believe that Crohn's disease has an autoimmune origin, related to self-reactive T cells or autoantibodies. This is largely based on the observation of extensive T cell infiltration and activation within established lesions. In general, autoimmunity can arise through a number of mechanisms. Central tolerance can prove inadequate, for example, if antigens expressed in thymus are subjected to different post-translational modifications in the periphery generating neo-epitopes with the potential to drive T cell activation[164]. The inherent randomness of somatic mutation and gene rearrangement of recognition motifs in lymphocytes in secondary lymphoid organs entails a risk of creating autoreactive cells. Defects in autoantigen clearance or T_{reg} function have also been postulated[162]. Finally, neo-antigens can arise through the release of previously sequestered proteins, by molecular mimicry (antigen-specific) or bystander activation (cytokine driven and not antigen-specific), leading to expansion of a population of low affinity autoreactive cells that have escaped negative selection. An infective influence is frequently implicated but rarely proven in initiating the latter two mechanisms.

Although reviews in the literature frequently refer to Crohn's disease as an autoimmune disorder, it is not at all clear that this is the case under the strictest definitions[170]. These include the positive criteria that patients should possess pathogenic autoreactive T cells or autoantibodies, a human leukocyte antigen (HLA) association, and that there should be a convincing animal model of the disease (see *1.8*). The negative criterion of no alternative explanation also applies.

1.4.3 Antibodies

The role of antibodies in establishing Crohn's lesions remains similarly unresolved. Abnormalities in immunoglobulin production have been documented, including an increase in serum IgG subtypes[171]. Antibodies against *Saccharomyces cerevisiae* are associated with Crohn's disease[172], and segregate with disease concordance in monozygotic twins[173]. These could, however, arise from raised exposure to bowel contents consequent to increased permeability of the bowel wall occurring as a result of active inflammation. Their pathogenic capacity remains dubious, although in theory they could cross-react with host antigens or lead to autoreactivity through epitope spreading.

It is possible that some of the extra-intestinal manifestations of Crohn's disease, most notably the arthritides[174], are secondary to induced autoantibodies arising following increased exposure to gastrointestinal microbes that have translocated across the mucosal barrier. Again, these could be directed against previously sequestered antigens, neo-antigens created by a microbial hapten, or cross-reacting self-antigens.

1.4.4 The hygiene hypothesis

The hygiene hypothesis is often invoked to explain the increase in incidence in Crohn's disease over the last few decades of the previous century[5], its predominance in the Western world and its scarcity in the tropics and other regions with high rates of endemic intestinal infection and infestation[175]. In general terms, the hypothesis proposes that naturally occurring microbial exposures, particularly in childhood, immunize against future development of allergy and autoimmune disease, and that increased standards of cleanliness impair this protection[176].

The rise in Crohn's disease in particular has been temporally correlated with the widespread introduction of domestic refrigeration[177] and was found to associate with the presence of better household amenities in infancy[178]. Appendectomy has also been identified as a risk factor[179], although the theory that this results in impaired peripheral T cell suppression remains speculative. Finally, this could explain the beneficial therapeutic effects of helminths in Crohn's disease reported in early clinical trials[180]. No cause and effect relationship, however, has been established for any disease, and interpretation is limited in view of the many potential confounding influences. Whilst the hygiene hypothesis remains attractive, it should not be accepted uncritically.

In summary, although established Crohn's lesions are characterised by substantial activation of cells of the adaptive immune system, the direct evidence for causality remains weak and rather circumstantial. The involvement of lymphocytes could be secondary to alternative underlying processes.

1.5 The innate immune system

1.5.1 Mucosal integrity is reduced in Crohn's disease

The normal intestinal mucosa does not constitute a complete barrier to luminal contents: M cells interrupt the enterocyte layer and pass luminal contents to leukocytes in the submucosa (see *1.2.3*), thereby permitting limited entry of luminal contents into the bowel wall. Whether the bowel in Crohn's patients allows greater permeation has come under scrutiny. Studies of intestinal permeability typically rely on oral administration of test probes *in vivo* and subsequent measurement of their secretion in urine[181]. Compounds employed are typically molecules with minimal uptake or metabolism in normal bowel, usually poorly digested carbohydrates or radiolabeled chelators. Although many early studies relied on a single test probe, this remains suboptimal as urinary accumulation depends not only on intestinal uptake but also the rate of gastric emptying, luminal degradation, volume of distribution and renal function. Studies with an improved design also examined the ratio between probes of differing permeabilities, which should be affected identically by extra-mucosal factors.

Most studies of intestinal permeability have demonstrated some increase in Crohn's patients to probes such as polyethyleneglycol (PEG)[182] lactulose/mannitol[183], [^{51}Cr]-ethylenediaminetetraacetic acid (EDTA)[184] and [^{99m}Tc]-diethylenetriaminopentaacetic acid (DTPA)[185], although a few have failed to replicate these findings[186]. Permeability was increased even in patients with quiescent disease and macroscopically normal bowel[187], but more so in those with active inflammation. There was no difference between subjects with small or large bowel involvement, and some enhancement was also observed in

ulcerative colitis. One case report describes an increase in a healthy individual with a macroscopically normal; they manifested Crohn's disease 8 years later[188].

Permeability was also found to provide an excellent predicator of relapse[189]. Interestingly, a parallel increase in pulmonary mucosa absorption of [^{99m}Tc]-DTPA has been documented in Crohn's patients[190] as has a similarly increased intestinal PEG uptake in their unaffected relatives[191]. Although others have failed to confirm the latter finding[192-194], many of the negative studies do show a non-significant rise and may have been underpowered to detect a true abnormality[181]. These last observations, in conjugation with the defect occurring in inactive disease, argue that this phenomenon may play a primary role in disease pathogenesis that becomes compounded by active inflammation.

The same phenomenon has also been assessed *in vitro* in resected mucosa by determining the transmucosal permeation of fluorescent ovalbumin and dextran using confocal microscopy[195]. Ovalbumin uptake was increased in non-inflamed specimens from Crohn's subjects compared to cancer patient controls, although dextran permeation was equivalent between the two groups. Ovalbumin translocated via both the transcellular and paracellular routes; dextran only by the former. This implies a derangement in intercellular tight junctions, which conforms with previous observations in non-inflamed Crohn's bowel in which the protein filaments comprising these structures were fragmented and disarrayed into irregularly distributed strands[196]. Crohn's bowel is also more sensitive to increases in permeability induced by photo-activated C10, an agent known to disrupt tight junctions[197]. This correlated with increased paracellular permeability to [^{51}Cr]-EDTA, tight junction dilatation on electron microscopy and more pronounced disassembly of peri-junctional F-actin.

Alterations in the mucus barrier have also been implicated. Polymorphisms in the *MUC3A* gene may increase susceptibility to Crohn's disease[198]. The expression of several other mucin genes has been suggested to be altered in both normal and diseased tissues[199-201], as have the post-translation modifications of the proteins[202], altering both the quantity[203] and consistency of the gel layer. A reduction in mucosal integrity, as highlighted by these studies, could predispose to increased ingress of bacteria and other luminal constituents into the bowel wall, as occurs in the cadherin dominant-negative mouse[36] (see *1.2.3*). This would place the mucosal immune system under greater stress to clear the exogenous material.

1.5.2 Acute inflammation is required to remove debris from the bowel wall

Any debris that accumulates in the bowel wall must be removed to prevent its accumulation and, in the case of microorganisms, collateral spread and infection. This is largely achieved by phagocytosis. Tissue macrophages are highly phagocytic but their respiratory burst capacity (see *1.5.3*) undergoes down-regulation[204]. Neutrophils remain the most efficient phagocytes, and provide an early defence after bacteria, fungi or other organic debris have breached the mucosal barrier[205]. They have a short lifespan and are normally sparse in the intestinal lamina propria; hence they must be recruited from the surrounding microcirculation. Microbial invasion provokes the release of cytokines and vasoactive factors by resident mucosal leukocytes, eliciting influx of neutrophils and further mononuclear cells.

Mice in which both the gp91 component of the NADPH oxidase enzyme complex and inducible nitric oxide synthase (iNOS) have been knocked out

demonstrate that neutrophils are critical for homeostasis in this manner[206]. The bactericidal capacity of these phagocytes is almost completely abrogated. The most apparent phenotype is the development of multiple abscesses, predominantly abdominal, containing organisms derived from the normal gut flora.

Furthermore, despite its proposed central role in driving Crohn's disease, evidence from murine models suggests that TNF-α may protect against prolonged intestinal inflammation. In the DSS model (see *1.8.1*), blockade of TNF-α aggravated the development of acute colitis, whilst ameliorating inflammation when administered at a chronic established stage[207]. This is reminiscent of two case-reports in which arthritis patients started on Etanercept (see *1.1.2*) developed Crohn's disease[208,209]. Additionally, mice deficient in TNF-α appear more susceptible to acute colitis induced by DSS, with a 60% 7-day mortality compared to 0% in wild type animals[210], similar to the TLR-4 knockout animals[97] (see *1.2.6*). These studies suggest that activation of pro-inflammatory mechanisms early after mucosal insult is essential to remove potential noxious agents and prevent the development of a chronic inflammatory state.

1.5.3 Colitis in chronic granulomatous disease

The key importance of neutrophils in mucosal immunity is underscored by a number of congenital diseases characterised by compromised neutrophil function, of which chronic granulomatous disease (CGD) represents the prototype. CGD is a rare genetic disorder, with a prevalence of approximately 1 in 250,000[211], caused by mutations in proteins that form or interact with the NADPH oxidase enzyme complex[212]. The ability of neutrophils to kill and digest

bacteria depends critically on the respiratory burst, which is defective in CGD. This process consumes oxygen by reducing it to O_2^- within the phagocytic vacuole, mediated by the import of electrons by NADPH oxidase.

It was commonly thought that reactive oxygen species (ROS) generated in this way are directly bactericidal, although this theory has recently been questioned[213]. During the respiratory burst, cytoplasmic granules containing digestive proteases discharge their contents into the vacuole. Mice deficient in these enzymes but with intact free radical generation show a major impairment in microbial killing.

Patients with CGD have an absent or greatly attenuated respiratory burst, with consequent impairment in innate immunity. They present with recurrent, severe bacterial and fungal infections, and chronic granulomatous inflammation involving many organs, but most frequently in the bowel and cervical lymph nodes[214]. The majority of patients are diagnosed in infancy. Until thirty years ago, death during childhood was common. Now, due to increased awareness of the disease and prophylaxis with antibiotics[211], a higher prevalence of its chronic sequelae has emerged.

The bowel disease found in CGD is remarkably similar to that of Crohn's disease; indeed a significant proportion are mistakenly diagnosed as such before the recognition of multiple infections[215]. In both, chronic enteritis occurs, usually separated by skip lesions of normal mucosa. Ulceration extends to the submucosa with frequent fissuring and fistulation, and peri-anal disease is often present[216]. Gastrointestinal manifestations reported in a recent review of the US registry of CGD patients included peri-anal abscess formation in 13% of patients, colitis in 17%, and granulomatous obstruction at the gastric pylorus or cardia in 15%[211].

Even higher levels of bowel involvement have been described in other case series[214]. These might still be underestimates: no study has examined the rates of endoscopic disease, and subclinical small intestinal malabsorption may occur in the majority of patients[217]. The reasons behind the phenotypic heterogeneity of the bowel disease remain largely unresolved, although polymorphisms in the myeloperoxidase gene have been associated with peri-anal involvement[218].

Macroscopic and histological features of CGD colitis appear largely comparable to Crohn's disease[217,219]. Microscopic characteristics include ulceration of the colon with the formation of granulomata, comprised of sharply defined aggregates of epithelioid histiocytes surrounded by a dense cuff of lymphocytes[219]. Subtle differences have been identified, such as the nature of the histiocytes (which are lightly pigmented and lipid-laden), a paucity of neutrophils, and multiple degranulating eosinophils within the crypts[220].

Clinical therapeutic trials for CGD enteritis are limited due to the rarity of the disease. In a double-blind, placebo-controlled study involving 128 patients, recombinant human IFN-γ was effective, although the study was underpowered to determine its prophylactic capacity[221]. Recombinant human G-CSF administration has been associated with colitis remission[222]. Non-myeloablative, matched bone marrow transplantation can successfully reduce the incidence of gastrointestinal granulomata[223], and exogenous neutrophil transfusion has been used in CGD to treat life-threatening infections[224]. As with Crohn's disease, immunosuppressive agents (including 5-aminosalicylate drugs, steroids, azathioprine and Infliximab) also relieve symptoms[219].

The important principle established by CGD enteritis is that the pathological picture observed in Crohn's disease can develop as a consequence

of impaired digestion by neutrophils. This may not only inform on pathogenic mechanisms but also point to novel therapeutic approaches.

1.5.4 Defects in neutrophil recruitment in Crohn's disease

Although Crohn's disease presents as a condition characterised by excessive inflammation, mounting evidence suggests that these patients in fact possess an impaired acute inflammatory response. The proposition is that clearance of bacteria and other organic debris from the bowel wall is consequently suboptimal, leading to its persistence (Fig. 1.5). The latter could elicit granuloma formation as a protective response, to limit collateral spread of potentially pathogenic material. Since the granuloma consist of activated macrophages and lymphocytes, subsequent release of pro-inflammatory cytokines such as TNF-α could drive the secondary, chronic inflammation found in active disease.

This theory could explain the relative organ specificity of Crohn's disease, predominating in the gut due to its high bacterial load. It could potentially account for the increased incidences of various bacteria reported in this condition (see *1.3.2*), resulting not from higher infection rates but impaired clearance. The similar pathology that occurs in CGD enteritis (see *1.5.3*) sets an important precedent for such a mechanism, as do the colitides also described in other neutrophil disorders including glycogen storage disease-Ib[225], leukocyte adhesion deficiency[226], Chediak-Higashi syndrome[227], and a variety of neutropaenias[228-231].

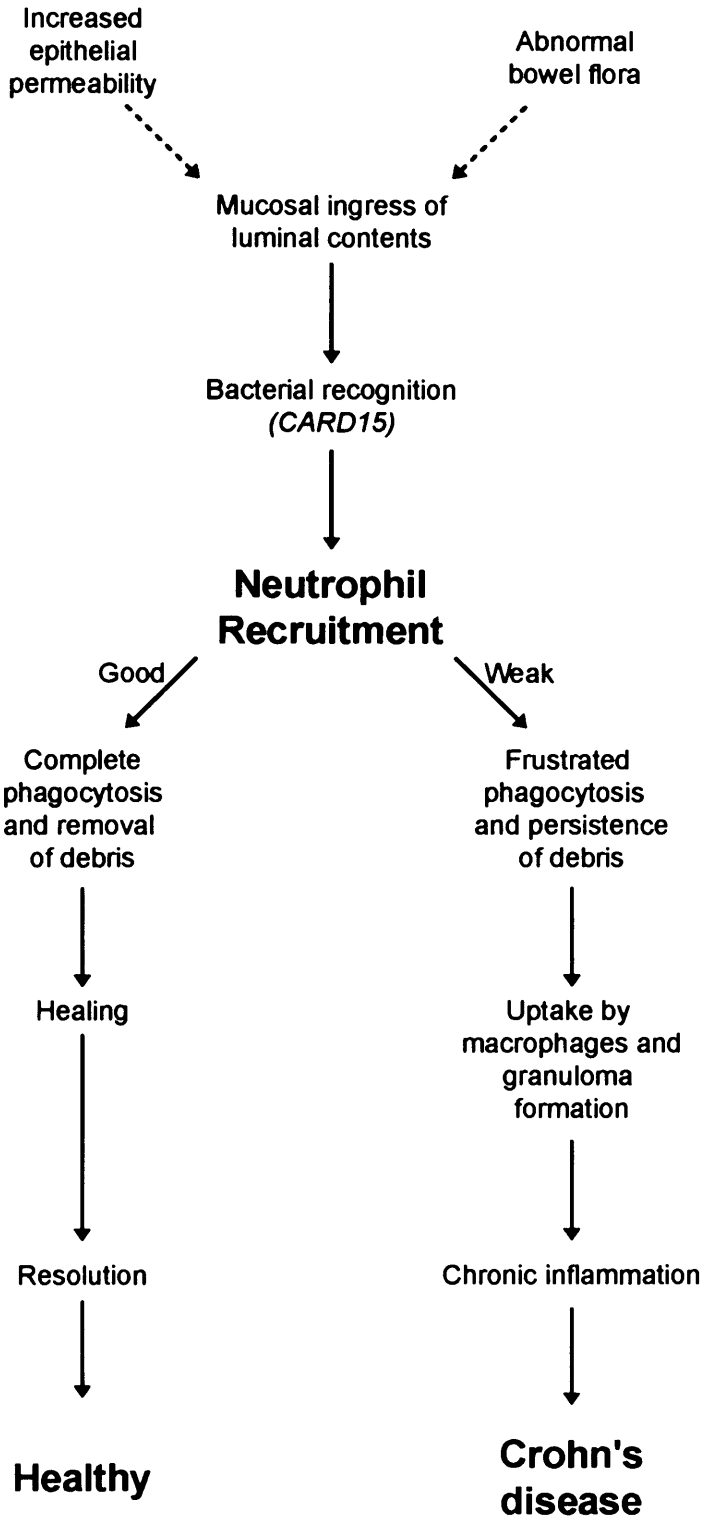


Figure 1.5 Flowchart illustrating the hypothesis of defective neutrophil recruitment. This process is normally required to remove exogenous material from the bowel wall. Its impairment may lead to Crohn's disease through the persistence of debris and a granulomatous reaction. The documented changes in the intestinal flora and epithelial permeability (dotted arrows) and *CARD15* polymorphisms may feed into this scheme by further stressing the system.

Neutrophil biology in Crohn's disease has previously been investigated under this hypothesis. A significant abnormality was discovered, in that Crohn's patients exhibited markedly delayed recruitment of neutrophils *in vivo* to the sites of breaches in an epithelial layer, as demonstrated in the skin window model of acute inflammation[232]. This defect did not occur in inflammatory disease controls, nor did it relate to disease activity or use of medication. The neutrophils responded normally under *in vitro* assays of chemotaxis[233,234], suggesting an alteration in the inflammatory milieu rather than an intrinsic failure of leukocyte motility. A circulating inhibitor of inflammation has been postulated on the basis of *ex vivo* experiments[235]: serum from inflammatory bowel disease patients (in a non-disease specific manner) inhibited *in vitro* chemotaxis to casein to a greater degree than that from healthy controls. The inhibitory factor was never formally identified, although its biochemical properties promote immunoglobulins as probable candidates. This abnormality, however, remains unproven as the cause of depressed neutrophil chemotaxis *in vivo* in Crohn's disease.

Defects have also been reported in other areas of neutrophil biology, although the data are less consistent. These include a reduced ability to generate superoxide in both active and quiescent disease[236], and attenuated phagocytic and bactericidal capacities[237].

Granulocyte/macrophage-colony stimulating factor (GM-CSF) activates neutrophils and augments their production and release from bone marrow. A double-blind, randomized control trial of GM-CSF in 124 patients with moderately to severely active Crohn's disease found some evidence of treatment benefit[238]. This may, however, have studied a suboptimal patient group, as such

agents may be more suited to maintenance of remission. The therapeutic efficacy of elemental diets, which might reduce intestinal bacterial loads, and helminths could also be explained under this hypothesis (see *1.4.4*). The effects of the latter are usually attributed to their augmentation of Th2 responses that reciprocally suppress the Th1 inflammation observed in active Crohn's lesions; instead they could prime the innate immune system for subsequent mucosal breach.

1.5.5 Mononuclear phagocytes in active Crohn's disease

The function of monocytes and macrophages remains less well investigated in Crohn's disease. Macrophages are activated in established lesions[239,240], although this may represent a secondary process unrelated to their generation. Monocytes from patients contain increased concentrations of lysozyme[241], and may exhibit increased respiratory burst activity[242,243] and increased antigen presentation to T cells[244], but these results directly correlate with disease activity. Any future investigation of their function will require careful controls to delineate primary defects from those due to an active systemic acute phase response or use of medication. There is an increasing realisation that mononuclear phagocytes might play a central role in the disease pathogenesis following the discovery that polymorphisms in *CARD15* (see *1.6*), expressed in these cells, predispose to Crohn's disease.

1.5.6 Resolution of inflammation

The termination of an inflammatory reaction requires that pro-inflammatory signals are discontinued and wound healing, for example of an ulcerated mucosal surface, commences[245,246]. The previous view that inflammation simply switches

off, possibly due to catabolism of the driving stimuli, is over-simplistic. Some of the regulatory cells and cytokines involved in negative regulation have been discussed previously (see *1.2.5*). Indeed, a murine model of colitis can be prevented or cured by the adoptive transfer of $CD4^+CD25^+$ T_{reg} lymphocytes, through mechanisms involving IL-10 and TGF-β[247,248].

A number of novel mechanisms involved in the control of inflammation are currently being elucidated. These include a family of lipid mediators generated by an alternative metabolic pathway downstream of cyclooxygenase (COX) and lipooxygenase (LOX), the enzymes responsible for the synthesis of pro-inflammatory prostaglandins and leukotrienes[249]. The lipoxins are generated by transcellular metabolism of arachidonic acid, requiring intercellular conversion of intermediary lipids[245,250]. The resolvins are produced by the action of COX and LOX on alternative substrates, eicosapentaenoic and docosahexaenoic acids[251]. These compounds have a number of anti-inflammatory effects, including inhibiting leukocyte trafficking[250] and pro-inflammatory cytokine release[252].

Another recent discovery concerns NF-κB, previously assumed to exert only a pro-inflammatory influence. NF-κB is a generic term for a family of dimeric transcription factors formed by heterodimerization or homomeric associations of the 5 members of the Rel family[253]. In the early stages of the inflammatory response, the principal NF-κB isoform consists of the c-Rel-p50 heterodimer[254], which promotes the transcription of many pro-inflammatory genes including TNF-α, IFN-γ[255], and iNOS[256]. In resolution, the predominant form changes to p50-p50 homodimers that possess distinct DNA-binding sites[257];

these induce anti-inflammatory cytokines, including TGF-β, and pro-apoptotic caspases[255].

Recruited macrophages also play a critical role in resolution: they remove apoptotic neutrophils by phagocytosis[258] and secrete metalloproteinases and other mediators that stimulate remodelling of the extracellular matrix, promoted by factors such as epidermal growth factor (EGF) and TGF-β[246]. The latter, secreted in part by neutrophils, also attracts myofibroblasts that produce collagen, fibronectin and other extracellular matrix components. Capillary endothelial cells then migrate into the region to provide a new blood supply.

Studies of tissue repair in Crohn's disease remain at a preliminary stage. Impaired wound healing has been reported post-operatively[259], and these patients develop intestinal fibrosis and fistulae indicative of such a problem. Fibroblasts from Crohn's strictures preferentially secrete TGF-β_2[260], an isoform thought inefficient in promoting wound repair perhaps by producing excess type III collagen, although its significance is still unclear.

1.6 CARD15

1.6.1 Hereditary susceptibility to Crohn's disease

A hereditary component to Crohn's disease has long been recognised[16]: monozygotic twins show approximately 50% disease concordance, the condition can cluster in families, and about 15% of patients have an affected relative. Pedigree analyses suggest polygenic inheritance with a significant environmental input acting upon a susceptible genetic background.

1.6.2 CARD15 polymorphisms predispose to ileal Crohn's disease

In 1996, a genome-wide linkage screen for inflammatory bowel disease susceptibility loci highlighted the *IBD1* region on chromosome 16q12[261], a finding supported in subsequent linkage studies[262,263]. Over the next five years, the same group progressively refined linkage maps to the region. In 2001, they tracked down single nucleotide polymorphisms (SNPs) in the *CARD15* gene (originally named *NOD2*), which accounted for the majority of this linkage[264]. At the same time, a second group also identified *CARD15* as the *IBD1* susceptibility gene using a candidate gene approach[265].

Three polymorphisms in *CARD15* predispose to Crohn's disease: two missense substitutions (R702W, G908R) and a single base-pair insertion (L3020fmsC)[264]. In Caucasian populations, approximately 30% of Crohn's patients carry one of these variants on a single allele (compared to 15% of healthy controls; odds ratio $\approx$ 3; Fig. 1.6). In contrast, approximately 15% of Crohn's patients are homozygous or compound heterozygous for these variants (odds ratio $\approx$ 40), whereas it is rare for healthy individuals to carry two polymorphisms although some individuals have been reported[266]. In addition to these polymorphisms, a number of other rare mutations in *CARD15* co-segregate with Crohn's disease in individual families[267], but do not achieve a population frequency of 1% required for definition as a polymorphism. *CARD15* seems to play a role mainly in Caucasian populations, in which the association has been widely replicated although frequencies show some variability[268-272]; the variants are rarely seen in Chinese[273] or Japanese[274] patients.

Several studies have attempted to define the impact of *CARD15* variants on clinical phenotype. The only consistent finding is that patients with these

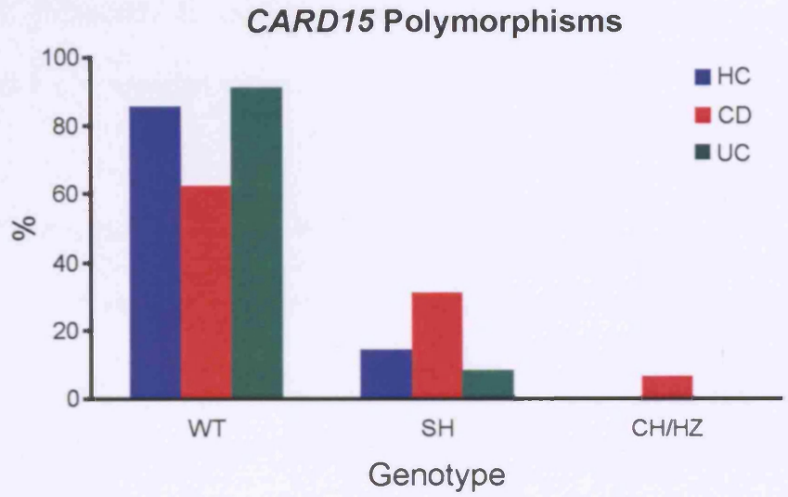


Figure 1.6 *CARD15* polymorphisms. These are more prevalent in patients with Crohn's disease (CD) than in healthy controls (HC) or patients with ulcerative colitis (UC). Carriage of two polymorphisms strongly, but not inevitably, predisposes to CD. Graph adapted from data in ref. 264.

WT: wild type; SH: simple heterozygous; CH: compound heterozygous; HZ: homozygous

polymorphisms almost exclusively develop disease involving the ileum[275]. The mechanism behind this localisation remains poorly understood. Some studies have reported a predisposition to fibrostenotic disease with a younger age of onset[267,276], although this is not universally replicated[8,268] and was not independent of ileal location. Differences may also occur because of other confounders in the populations studied, such as duration of disease or alternative treatment protocols in the various centres. The polymorphisms do not seem to occur more frequently in patients with a positive family history of Crohn's disease or in those reported to have granulomata on biopsy[277,278].

1.6.3 CARD15 responds to bacterial cell wall products

CARD15 is normally expressed predominantly in monocytes and macrophages[279,280]. It has also been reported in neutrophils[281], intestinal Paneth cells[282] and, under inflammatory conditions, within intestinal epithelial cells[280]. It encodes a cytoplasmic protein, whose structural domains include two caspase-recruitment domains (CARDs), which mediate protein-protein interactions; a central nucleotide-binding domain (NBD) important for activation; and ten C-terminal leucine-rich repeats (LRR) thought to bind ligands[279] (Fig. 1.7). All polymorphisms associated with Crohn's disease occur within or near the LRR; the L3020finsC change produces a premature STOP codon and a truncated protein[265].

CARD15 was discovered through database searches for proteins with homology to Apaf-1 (the basic subunit of the apoptosome) and CARD4 (see *1.7.2*)[279]. The crystal structure and molecular mechanism of activation of Apaf-1 is well described[283]. It possesses one N-terminal CARD, a central NBD, and C-

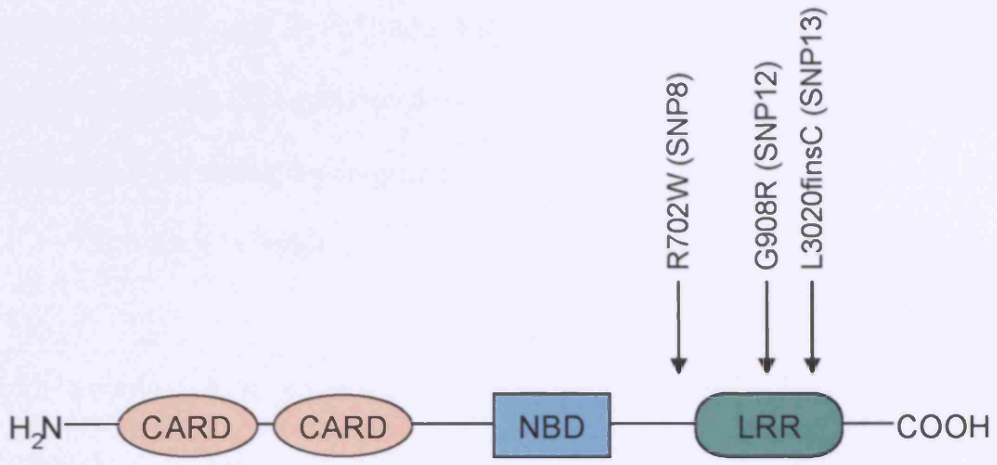


Figure 1.7 Schematic diagram of the structure of CARD15. The protein contains two N-terminal Caspase-Recruitment Domains (CARDs), a central Nucleotide-Binding Domain (NBD), and C-terminal Leucine Rich Repeats (LRRs). The polymorphisms associated with Crohn's disease occur in or near the LRR.

terminal WD-40 repeats. The latter acts as a recognition domain for mitochondrial damage through the binding of cytochrome c[284]. Under basal conditions, these repeats exert an inhibitory influence on the rest of the protein[285]. This is released on ligand binding, leading to Apaf-1 oligomerization to form the heptameric complex that comprises the apoptosome[283]. This binds procaspase-9 through homophilic interactions between the CARDs of the respective proteins. The induced proximity then leads to proteolytic activation of procaspase-9, initiating the caspase cascade and programmed cell death.

In contrast to Apaf-1, CARD4 was found not to contribute significantly to apoptosis but potentially to act as a pathogen recognition receptor,[286] based partly on its homology to R proteins important in plant immunity[287]. It was originally suggested that CARD15 could act in a similar manner by binding LPS[279]. This was an attractive proposal in view of the proximity of Crohn's lesions to areas of the gut with the highest bacterial colonization. Transfection of HEK293 cells with wild type *CARD15* conferred responsiveness to LPS, resulting in activation of NF-κB[265] and IL-1β transcription. Subsequent purification of the LPS preparation revealed that this activation actually derived from contaminating muramyl dipeptide (MDP), a component of bacterial cell wall peptidoglycan[288,289].

To determine the role of the various domains of CARD15, cells were transfected with a panel of mutants[279]. NF-κB activation was enhanced by deletion of the whole LRR, consistent with an auto-inhibitory function similar to the WD-40 repeats of Apaf-1, which is removed on ligand binding. Deletion of either CARD was sufficient to abrogate activity, suggesting these represent the effector domains, which bind and activate the CARD-containing serine/threonine

kinase RIP-like interacting CLARP kinase (RICK, also called RIP2 or CARDIAK).

Whereas cells with wild type *CARD15* activate NF-κB on stimulation, those transfected instead with a gene containing mutations associated with Crohn's disease showed a greatly attenuated response[265,288]. This presents a paradox, as leukocytes in active Crohn's lesions exhibit markedly increased NF-κB activity[239,290], and preliminary evidence suggests that inhibitors of this transcription factor might ameliorate the inflammation[291].

Four principal hypotheses have therefore arisen to explain how *CARD15* polymorphisms confer susceptibility to Crohn's disease:

- That exposure to MDP fails to activate anti-inflammatory mediators[292];
- That failure to respond normally to MDP leads to an exaggerated adaptive immune response[265];
- That loss of CARD15 in the gastrointestinal tract leads to deranged mucosal immune homeostasis[282]; or
- That there is a toxic gain of function[293].

There is some evidence to support each of these theories. Stimulation of macrophages with MDP does induce anti-inflammatory IL-10 secretion[292]. CARD15 has been reported to negatively regulate NF-κB signalling in response to Toll-like Receptor-2 (TLR2) activation[294], although this has not been universally replicable[295]. CARD15 has also been documented in the intestine within epithelial cells and in particular within Paneth cells[282], in which patients carrying the polymorphisms exhibit diminished expression of α-defensins[296].

This might be predicted to lead to expansion of the bacterial flora, with the caveat that direct evidence remains lacking[297] and mice deficient for all mature Paneth cell defensins exhibit no overt intestinal pathology or microbial overgrowth[298]. Alternatively, intestinal epithelial cells transfected with wild type *CARD15* show enhanced containment of *Salmonella typhimurium* infection compared to those transfected with mutant genes[299]. Finally, a gain of function is supported by a recent mouse model (see *1.6.4*)[293].

One last hypothesis proposes that CARD15 interacts through its CARD domain with members of the caspase cascade[300]. Many of these proteins are involved in apoptosis, and failure to initiate programmed cell death could result in the accumulation of activated leukocytes within the bowel wall. Others, such as caspase-1, form part of the inflammasome. This intracellular complex processes and activates cytokines including IL-1β[301], and could be aberrant in Crohn's disease. Experimental evidence is still required to assess the merits of these last theories.

1.6.4 CARD15 murine models

The murine homologue of *CARD15* is encoded on chromosome 8[302]. It displays 78% sequence homology with the human gene, and possesses similar structural motifs. As with the human gene, predominant expression occurs in peripheral blood leukocytes and resident tissue macrophages, with lower levels in spleen, granulocytes and dendritic cells. When the wild type gene was transfected into HEK293 cells, MDP exposure activated of NF-κB. The nucleic acid sequences at the equivalents of two of the human polymorphic sites, G908R and L3020finsC, are conserved in the mouse[303].

Mice have been generated in which *CARD15* has either been knocked out[295], or contains G881R or L980finsC variants that correspond to human G908R or L3020finsC respectively[302]. All demonstrate an absent or attenuated response to MDP, but not other bacterial derivatives. In mice carrying the L980finsC, mutant protein expression is lower but not sufficiently to explain the degree of reduced activity. None of these animals spontaneously developed intestinal pathology.

To attempt to elicit a phenotype, the knockout mice were exposed to various bacteria or their products[295]. There was no difference in the response to an endotoxic shock model, apart from when the mice were first sensitized by injection with MDP, in which case the knockout animals exhibited a survival advantage. Immunization of the mice with intra-peritoneal MDP led to considerable IgG1 generation in wild type animals but not in the knockouts; this was highlighted as providing a link to the adaptive immune system. The mice were then challenged with the intracellular gram-negative bacterium *Listeria monocytogenes*. There was no discernable difference with intravenous or intra-peritoneal inoculation, although knockout animals were more susceptible following intra-gastric exposure. This was suggested to indicate that CARD15 plays a specific and pivotal role in protecting against bacterial infection in the intestine, with possible mechanisms including recognition of bacteria by enterocytes, Paneth cell function and production of α-defensins.

A transgenic mouse was recently generated carrying a mutation in *CARD15* that results in a truncated protein lacking the last 33 amino acids, similar to the human L3020finsC variant[293]. In contrast to data from humans and transfected cells lines, macrophages from these mice demonstrated elevated NF-

κB and IL-1β activation on stimulation with MDP. These mice also failed to develop spontaneous intestinal inflammation, but in the DSS model (see *1.8.1*), they demonstrated increased weight loss and mortality, with more extensive inflammatory lesions in the colon and higher NF-κB activation and cytokine expression. Oral antibiotics protected against this phenotype. Although these mice display an abnormally exuberant inflammatory response, they present a quandary since the functional effects of the mutations are in the opposite direction to those that predispose to Crohn's disease in humans. This controversy has yet to be resolved, although it is possible that it relates to the strain of mouse used or that the structural effects of the generated mutation do not correspond to the human variant as closely as predicted.

1.7 Alternative susceptibility genes

1.7.1 Loci identified by genome-wide linkage analysis

A number of other potential susceptibility loci for human inflammatory bowel disease have been identified by genome-wide linkage analyses. The *IBD2* locus on 12p13.2-12q24.1[304] predisposes specifically to ulcerative colitis[305]. The HLA region on 6p contributes susceptibility to *IBD3*[306], with several haplotypes potentially increasing the risk of Crohn's disease and HLA-B27 associated with the co-occurrence of ankylosing spondylitis[307]. The only other chromosomal location originally described by linkage for which the gene has been identified remains the *IBD5* locus (see *1.7.4*). Other regions still under investigation include *IBD4* on 14q11-q12[308], *IBD6* on 19p13[309], *IBD7* on 1p36[310], *IBD8* on 16p[311] and *IBD9* on 3p26[312]. The majority of the remaining potential susceptibility genes reported were chosen based on a candidate gene approach.

1.7.2 CARD4

Given the association between polymorphisms in *CARD15* and Crohn's disease, *CARD4* (originally named *NOD1*) presented an obvious candidate gene. This is located on chromosome 7p14.3[313], a region of known linkage to inflammatory bowel disease[304]. Although an early study found no associations[314], a more recent investigation reported a complex deletion allele called ND_1+32656*1 that co-segregated with Crohn's disease[315]. This was weakly associated with the presence of extraintestinal manifestations but no other disease feature.

CARD4 was also originally found on the basis of homology to Apaf-1[313]. It possesses a single N-terminal CARD, a central NBD, and ten C-terminal LRRs. It encodes a cytosolic protein, expressed ubiquitously rather than predominantly in myeloid cells. As with the CARD15 protein, it was initially believed to bind LPS and activate RICK, NF-κB and IL-1β production[286], but this too was mediated by a peptidoglycan contaminant, N-acetylglucosamine-N-acetylmuramic acid[316]. The mechanism by which the *CARD4* mutation predisposes to Crohn's disease likewise remains unresolved, though similar suggestions have been put forward. Evidence remains minimal, however, except that the wild type protein confers some protection against invasive intracellular bacteria such as *Shigella flexeneri*[317].

1.7.3 Other pathogen recognition receptors

A number of other receptors are involved in the sensing of pathogens, both on leukocytes and epithelial cells. The detection of LPS requires its dissociation from bacterial cells walls by serum lipopolysaccharide-binding protein (LBP)[318],

which also augments its presentation to the CD14 receptor on the external aspect of the plasma membrane[319]. CD14 has no transmembrane domain and must associate with TLR4[320] and an accessory molecule MD-2[321] in order to transduce the signal to the cytoplasm. Here, the MyD88 adaptor protein initiates an intracellular signalling cascade[322].

Polymorphisms in genes encoding two of these proteins have been associated with Crohn's disease: a T/C promoter variant at position -159 in *CD14*[323] and a D299G coding change in *TLR4*[324] that impairs the LPS response[325], although the latter occurs at similar frequencies in Crohn's disease and ulcerative colitis. Neither gene corresponds well with regions previously identified by linkage analysis.

A number of other Toll-like receptors have been investigated for variants that may predispose to inflammatory bowel disease but so far preliminary positive data exist only for Toll-like receptor-9 (*TLR9*), which detects double-stranded CpG-rich bacterial and viral DNA[326].

1.7.4 The IBD5 locus

Considerable excited was generated by the discovery of the *IBD5* locus on chromosome 5q31[327], due to its proximity to a cytokine gene cluster[328]. The underlying genes, however, have recently been identified and encode not inflammatory mediators but membrane transporters. Two polymorphisms have been reported to date: a missense L503F substitution in *SLC22A4* and a G/C transversion in the promoter region of *SLC22A5*[329]. Both predispose to Crohn's disease, particularly that with peri-anal involvement[330].

The genes encode proteins named OCTN1 and OCTN2 respectively[329]. These function as polytopic transmembrane organic cation transporters expressed in enterocytes, in which they localize to the apical brush-border membrane, macrophages and T lymphocytes. The polymorphism in *SLC22A4* maps to the eleventh transmembrane domain, known to be required for transport function. The promoter polymorphism leads to a reduction in the level of protein production. The mechanism by which these predispose to Crohn's disease remains highly speculative. Their substrates include carnitine[331], well known for its role in mitochondrial transport of fatty acids, but also a wide range of other organic cations including xenobiotics[329]. It is possible that variant proteins lead to disruption of the enterocyte barrier, or altered transport of microbial products.

1.7.5 Functional clustering of susceptibility genes

A number of other genes have been highlighted as potentially modifying susceptibility to Crohn's disease. Several of these encode cytokines or their related receptors and downstream signal transducers[332-338], and others influence the permeability of the gastrointestinal epithelial barrier[339,340]. The genes identified to date seem to fall into one of three functional categories: pathogen receptors; cytokines and related proteins; and molecules involved in maintaining epithelial integrity. The connection between these is that they determine the magnitude of the inflammatory response generated by intestinal bacteria.

1.8 Animal models of Crohn's disease

1.8.1 Chemically-induced colitides

A number of animal models of inflammatory bowel disease rely on the use of exogenous chemicals to induce colitis. These include acetic acid[341], phorbol esters[342], F-met-leu-phe[343], and sulfated polysaccharides such as carrageenan[344], amylopectin sulfate[345] and dextran sodium sulfate (DSS)[346]. These disrupt the epithelial barrier increasing mucosal exposure to the luminal flora; this leads to inflammatory reactions largely neutrophil mediated with relative lymphocyte independence[347]. Whilst these provide considerable information about mechanisms of mucosal immunity, they bear little pathological resemblance to Crohn's disease.

1.8.2 The IL-10 knockout mouse

IL-10 represents a prototypic anti-inflammatory cytokine, involved in inflammation resolution and negative regulation of the immune response[348]. Many have postulated that impairment in its production may underlie the chronic inflammation of Crohn's disease, and cite the IL-10 knockout mouse as a good model of the condition[349]. These animals develop a chronic enterocolitis, that can be prevented by rearing in a germfree environment[99] or with prophylactic antibiotics[350].

The inflammation, however, does not appear identical to Crohn's disease. Whilst leukocytes infiltrate the bowel in a transmural fashion, the process remains almost entirely confined to the large intestine[351]. Bowel involvement occurs in continuous segments rather than in skip lesions, without the formation of granulomata or any of the extraintestinal stigmata of human inflammatory

bowel disease. Its utility as a model of Crohn's disease can therefore be called in question.

This mouse model, however, illustrates two important points about the generation of intestinal inflammation. First, it demonstrates the requirement for the bowel flora in driving the process. It also implies that subclinical inflammation must normally occur in the bowel as a homeostatic process. This inference can be drawn from the function of IL-10 as an anti-inflammatory cytokine. Were inflammation not stimulated in the first instance in wild type animals, there would be no process to disinhibit in the knockout. It is highly likely, therefore, that low grade acute inflammation occurs physiologically in the gastrointestinal tract in order to remove any debris that accumulates within the bowel wall.

1.8.3 Other murine models

A number of other germ line models of intestinal inflammation have been generated; few resemble Crohn's disease. Two of the closest murine models are the $TNF^{\Delta ARE}$ and SAMP1/Yit mice. The former possesses a targeted deletion of AU-rich elements located in 3'-untranslated region of the *TNF-α* gene that dysregulates processing of its mRNA causing overproduction of the protein[352]. This mouse develops spontaneous mucosal inflammation, predominantly in the terminal ileum and occasionally in the proximal colon, characterised by transmural infiltrative lesions and granulomata; it also develops peripheral arthritis. The exact mechanism underlying this phenotype remains unclear but, as demonstrated by the variability in therapeutic efficacy of drugs targeting TNF-α

(see *1.1.2*) and murine models (see *1.5.2*), the matter is not as simple as the overproduction of a pro-inflammatory cytokine.

The SAMP1/Yit mouse also develops a granulomatous chronic inflammatory bowel disease[353]. These mice were produced through extensive inbreeding, and although the underlying defect remains unidentified, abnormal epithelial cell biology has been demonstrated.

1.9 Outline of the thesis

1.9.1 Summary of background information

The cause of Crohn's disease remains poorly understood but appears to relate to an abnormal interaction between the bowel luminal contents and the immune system. Most theories emphasize over-activation of the latter as the primary event. Conversely, Crohn's patients demonstrate reduced neutrophil recruitment after epithelial trauma in the skin. A consequent failure to clear bacteria and other debris from the bowel wall efficiently has been postulated, provoking a granulomatous reaction. The relevance of this abnormality to the gastrointestinal tract has never been demonstrated, and its molecular basis remains unresolved although factors extrinsic to the neutrophil itself appear important. Furthermore, the consequences in terms of the acute inflammatory response *in vivo* to bacteria have yet to be investigated.

The discovery of polymorphisms in the *CARD15* gene that confer susceptibility to Crohn's disease generated considerable excitement. Precisely how they contribute to the pathogenesis has stimulated considerable debate. The protein is normally found predominantly in mononuclear phagocytes, where it is thought to function as a receptor for bacterial MDP. The polymorphisms impair

its ability to activate NF-κB, but how this results in a pro-inflammatory state in Crohn's disease remains highly speculative.

1.9.2 Studies conducted in this thesis

To pursue the hypothesis that acute inflammation is diminished in Crohn's disease, four studies were conducted:

- The acute response to epithelial trauma in the gastrointestinal tract was examined in Crohn's patients and controls to determine whether the same defect applied to the bowel. A novel serial biopsy technique was developed for this purpose (see *Chapter 3*).
- To delineate the profile of acute inflammatory mediators produced and cellular activation states in control and Crohn's subjects, the skin window technique was modified to allow characterisation of most facets of the acute inflammatory response (see *Chapter 4*).
- Macrophage responses to various inflammatory stimuli were studied *in vitro* to try and identify the cellular and molecular bases for diminished responsiveness in Crohn's disease (see *Chapter 5*).
- The response to bacteria in the tissues was assessed directly *in vivo* by subcutaneous inoculation with killed *Escherichia coli* (see *Chapter 6*).

Throughout the *in vivo* studies, patients were carefully selected to avoid those taking (or who had recently stopped) immunosuppressive medications. Only subjects in remission were studied, to minimize potential confounding factors. Participants were genotyped for the *CARD15* polymorphisms known to

predispose to Crohn's disease, and their influence on the various parameters studied was determined.

1.9.3 Implications of these studies

The findings of these studies add considerable credence to the theory that the underlying abnormality in Crohn's disease relates to a weak initial inflammatory reaction. Although general mechanisms behind this impairment were elucidated, the precise molecular defects merit further investigation. Concepts regarding therapeutic maintenance of remission may also require some re-evaluation.

Chapter 2: Methods

2.1 Subjects

2.1.1 General note on chemicals and equipment

All chemicals and reagents were of the highest purity and purchased from Sigma Aldrich (MO, USA), and all reactions were conducted at room temperature, unless otherwise stated. All equipment use in cell culture experiments was sterile.

2.1.2 Subject selection

Healthy subjects were defined as individuals with no inflammatory disease and taking no immunosuppressive medication. Patients with a definitive diagnosis of Crohn's disease, confirmed by endoscopic, radiographic and/or histological appearances, were recruited from the gastroenterology outpatient clinic at University College London Hospital (UCLH). Standard diagnostic criteria were used to distinguish Crohn's disease from ulcerative colitis[354]: these included a discontinuous nature of mucosal involvement (particularly with ileal and peri-anal inflammation but relative sparing of the rectum); the formation of aphthous ulcers; mucin retention; and the presence of epithelioid granulomata. Subjects were excluded if the diagnosis had been made less than 1 year previously; if other causes of inflammation had not been excluded; if only non-specific features of inflammatory bowel disease were present; or if there was disagreement between the clinical and histological diagnoses. All patients had quiescent disease (see *2.1.4* for assessment). Patients were taking no immunosuppressive

medication (unless otherwise stated; these were in the minority) and control subjects were approximately matched for age, sex and smoking history. Patients with ulcerative colitis were recruited from the gastroenterology clinic and those with rheumatoid arthritis from the rheumatology outpatient clinic, under the same criteria. All studies were approved by the Joint UCL/UCLH Committee on the Ethics of Human Research (project numbers: 00/0004, 02/0324 and 04/Q0502/29). Written informed consent was obtained from all volunteers. Data were analyzed anonymously and blinded where possible.

2.1.3 Database of patient characteristics

A database was created in Microsoft® Office Access 2003 to record subject details, and registered and covered by the Data Protection Act 1998. For the Crohn's patients, information recorded included their age, sex, ethnicity and year of diagnosis; any previous intestinal resections; disease location and behaviour according the Vienna classification (inflammatory, fibrostenotic, fistulating or mixed fibrostenotic/fistulating)[355]; presence of granulomata on intestinal biopsy; *CARD15* genotype (see *2.2*); smoking history (current smoking activity and pack-year history); use of the oral contraceptive pill or hormone replacement therapy; use of medication, previous failed medications and reasons for failure; family history of inflammatory diseases; presence of extra-intestinal, upper gastrointestinal or peri-anal features of disease; disease activity (see *2.1.4*); and current co-morbidities or relevant past medical history. For patients with ulcerative colitis similar data was recorded, including extent of colorectal involvement.

2.1.4 Assessment of disease activity

The Harvey-Bradshaw score of disease activity[356] was calculated (Table 2.1), based on a single assessment of symptoms and signs pertaining to the preceding 24 hour period. This has been validated and shows good correlation with the more intensive Crohn's Disease Activity Index based on a 7 day diary[357]. Only patients with a Harvey-Bradshaw score <3 were studied. Peripheral venous blood was collected at the time of each experiment for measurement of white cell count, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) as systemic markers of inflammation. Serum albumin was measured as an indicator of the nutritional state[358], along with serum vitamin B_{12} and red cell folate where these were clinically indicated. All were measured as part of the routine clinical assessment through UCLH clinical haematology and biochemistry services. No patients with current fistulating disease were studied, although past development of fistulae or stenoses was documented.

2.2 *CARD15* genotyping

2.2.1 DNA extraction

Peripheral venous blood was collected by routine venesections into vacutainers containing ethylenediamine tetra-acetic acid (EDTA; Becton Dickinson, NJ, USA). Genomic DNA was isolated from 3 ml of these samples by magnetic separation using the GCT™ Genomic DNA Purification Kit (DNA Research Innovations Ltd, Kent, UK), following the instructions from the manufacturer. Briefly, blood was lysed and DNA recovered using magnetic beads, then purified by protease digestion followed by isopropanol precipitation.

General well-being	0 Very well 1 Slightly below par 2 Poor 3 Very poor 4 Terrible
Abdominal pain	0 None 1 Mild 2 Moderate 3 Severe
Number of liquid stools per day	1 Per episode -2 If previous bowel resection
Abdominal mass	0 None 1 Dubious 2 Definite 3 Definite and tender
Systemic Complications Arthralgia Uveitis Erythema nodosum Aphthous ulcers Pyoderma gangrenosum Anal fissure New fistula Abscess	1 per item

Table 2.1 Harvey-Bradshaw activity index.

2.2.2 Allelic discrimination

All patients were genotyped for the 3 *CARD15* SNPs associated with susceptibility to Crohn's disease: R702W, G908R and L3020fsinsC, previously reported as SNP8, SNP12 and SNP13 with reference SNP IDs rs2066844, rs2066845 and rs2066847 respectively.

Genotyping was conducted using the Taqman® SNP Genotyping Assays (Applied Biosystems, CA, USA) and ABI 7900HT Sequence Detection System (Applied Biosystems) on 5 ng/μl DNA, using allelic discrimination between probes for wild type and mutant alleles. Primers and probes for SNP12 were designed through the Custom Taqman® SNP Genotyping Assay (Applied Biosystems); those for SNP8 and SNP13 were taken from previously published papers[264,359,360] and were as follows:

SNP8 primer (forward):	5'-GCTGGCTGAGTGCCAGACATG-3'
SNP8 primer (reverse):	5'-AGTGGAAGTGCTTGCGGAGG-3'
SNP8 probe (wild type):	5'-CCTGCTCCGGCGCCAGGC-3'
SNP8 probe (mutant):	5'-CCTGCTCTGGCGCCAGGC-3'

SNP12 primer (forward):	5'-CTGTTGACTCTTTGGCCTTTTCAG-3'
SNP12 primer (reverse):	5'-CCACCTCAAGCTCTGGTGATC-3'
SNP12 probe (wild type):	5'-CTGTTGCCCCAGAAT-3'
SNP12 probe (mutant):	5'-CTGTTGCGCCAGAAT-3'

SNP13 primer (forward):	5'-GTCCAATAACTGCATCACCTACCT-3'
SNP13 primer (reverse):	5'-ACTTCCAGGATGGTGTCATTCC-3'

SNP13 probe (wild type): 5'-CTGCAGGCCCTTGA-3'

SNP13 probe (mutant): 5'-CTGCAGGCCCCTTGA-3'

A 1 µl aliquot of each sample was added to a clear optical reaction plate (Applied Biosystems) and left to dry overnight; wells without DNA were negative controls. A 5 µl mixture containing 2.5 µl Taqman® Universal Polymerase Chain Reaction (PCR) master mix (Applied Biosystems) and 0.25 µl of each primer in diethylpyrocarbonate (DEPC)-treated water (Ambion, TX, USA) was added to each well. The plate was sealed with optical adhesive covers (Applied Biosystems), spun down, and run in a MBS Satellite 384 Thermal Cycler PCR machine (Thermo, MA, USA) under the following conditions: 94°C for 30 s; 94°C for 30 s, 55°C for 30 s and 72°C for 30 s for 30 cycles; 72°C for 30 s, then held at 4°C. All samples were genotyped in duplicate. Any sample for which a conclusive genotype could not be established was directly sequenced.

2.2.3 Sequencing

The region of interest was amplified by PCR using 20 ng of genomic DNA template, 5 pmol of each primer, 200 µM dNTPs (Promega, WI, USA) and 1.25 U/reaction *Pfu* DNA polymerase (Promega). Primers used for sequencing (SNP12 was reliably genotyped by Taqman® in all cases) were ordered from MWG-Biotech AG (Ebersberg, Germany):

SNP8 primer (forward): 5'-CTCGGAGGGAAAGGACAGCAGCGTG-3'

SNP8 primer (reverse): 5'-CCAGCCGCTCCTCCTGCATCTCGTA-3'

SNP13 primer (forward): 5'-GGCTAACTCCTGCAGTCT-3'

SNP13 primer (reverse): 5'-TCCCGTCACCCCATTTTA-3'

Samples were run in a MBS Satellite 384 Thermal Cycler PCR machine under the following conditions: 95°C for 1 min; 95°C for 1 min, 61°C for 1 min (SNP8) or 50°C for 1 min (SNP13), and 72°C for 2 min for 35 cycles; 72°C for 10 min, then held at 4°C. PCR products were run on an agarose gel, purified using the Qiaex II gel purification kit (Qiagen, CA, USA) then sequenced using the BigDye v3.1 kit (Applied Biosystems), according to the instructions supplied by the manufacturer.

2.2.4 Analysis of genotype-phenotype correlations

The database was probed to examine phenotypic correlates of carriage of *CARD15* variants. Associations were determined using the chi-squared (χ^2) test with Fisher's exact test for discrete variables, calculated with Graphpad Instat version 3.00 for Windows ME (Graphpad Software, CA, USA). For continuous variables, the 2-tailed students' t-test was used for single comparisons and 1-way Kruskal-Wallis Analysis of Variance (ANOVA) with Dunn post-tests for comparisons between multiple groups to avoid errors associated with multiplicity. Both were calculated using Graphpad Prism version 4.01 (Graphpad Software). The threshold for significance was $P < 0.05$.

2.3 Serial intestinal biopsies

2.3.1 Biopsies

Biopsies were taken with 5 mm forceps from the posterior wall of the rectum in 7 non-inflammatory bowel disease controls, 5 Crohn's patients, and 3 patients with ulcerative colitis. A second biopsy was taken 6 h later under direct sigmoidoscopic visualization. In a few subjects, paired biopsies were taken from two adjacent sites in the rectum, both of which were re-biopsied, to evaluate the internal reproducibility of the technique. Paired serial biopsies were also taken from the rectum and neo-terminal ileum in a further 2 controls with familial adenomatous polyposis (FAP) and 3 Crohn's patients, all of whom had colectomies with ileorectal anastomoses.

2.3.2 Immunohistochemistry

Samples were fixed in formalin, embedded in paraffin wax and serially sectioned. These were then dewaxed, rehydrated and incubated with 3% H_2O_2 in methanol (VWR, Poole, UK) for 20 min to block endogenous peroxidase activity. Antigen retrieval was performed by pressure-cooking in 0.01 M citrate buffer pH 6.0 for 2 min at high pressure. The sections were incubated with serum-free Protein Block (Dako, Glostrup, Denmark) for 10 min then washed three times in TBST (Tris-buffered saline (TBS; 200 mM NaCl, 50 mM Tris-HCl pH 7.4), 0.5% (v/v) Tween-20).

Primary antibody was added for 1 h. Separate sections from each sample were treated with either monoclonal anti-human IL-8 (R&D Systems, MN, USA; 1:100) or anti-myeloperoxidase (Dako; 1:1,000) antibodies made up in TBS. Sections were washed 3 times with TBST then incubated with appropriate

biotinylated secondary antibodies (Dako; 1:300) for 30 min. Sections were washed again and incubated with streptavidin-peroxidase conjugate (Dako; 1:500) for 30 min, then developed with 3,3'-diaminobenzidine-tetrahydrochloride (Dako) and 0.1% (v/v) H_2O_2 solution for 2 min, counterstained with haematoxylin, and mounted with distrene/plasticizer/xylene (DPX) mountant. For negative controls, duplicate sections were used in which primary antibodies were omitted. Immunostaining was performed blinded to the patient identity and biopsy timing.

2.3.3 Analysis

The association between responses at paired sites in the rectum, or between the rectum and ileum, was determined using the Pearson correlation coefficient (the relationship appeared to be linear, and data assumed normally distributed). Calculations were performed on Microsoft® Office Excel 2003. Numbers of MPO-positive and IL-8-positive cells between different groups and under different conditions were compared using a 2-way ANOVA with Bonferroni post-tests (Graphpad Software). The threshold for significance was $P < 0.05$. Cell counting was performed in a blinded fashion by two independent observers.

2.4 Skin Windows

2.4.1 Creation of skin windows

Forearms were cleaned with 70% (v/v) ethanol (VWR). Skin windows were created by abrasion of a 3 cm x 1 cm area on the volar surface using grade C sandpaper (Homebase Ltd, Surrey, UK), until capillaries were visualized but before bleeding commenced. Lesions were always fashioned by the same

investigator, and for each subject it was confirmed by a second independent investigator that window sizes and duration and intensity of abrasion were uniform.

Abrasions were overlaid with sterile filter papers (Whatman Ltd., Maidstone, UK) saturated with injection-grade normal saline (B. Braun Medical Inc., PA, USA;0.9% w/v) either alone or containing MDP (100 ng/ml) or recombinant human IL-8 (PeproTech, NJ, USA; 10 μg/ml). Filter papers were then covered with a layer of Nescofilm sealing film (Karlan, AZ, USA) and an adhesive dressing. Dressings and filter papers were removed after either 30 min, 6 h, 24 h or 48 h depending on the experiment. Subsequently, windows were washed briefly with water then left open without a dressing to heal.

2.4.2 Cell differential counts

Filter papers were layered for 3 s on a glass slide. Adherent cells were fixed with methanol (VWR), stained by Romanowsky dye and differential leukocyte counts determined by morphology. Counts were taken in 5 randomly selected high power fields and mean values taken, with more than 150 cells counted in all control subjects.

2.4.3 Myeloperoxidase assay

Filter papers were then incubated in 400 μl normal saline on a rotating wheel (30 min, 4°C) to elute proteins. The filter paper and cells were separated from the fluid by centrifugation (15,000 *g*, 5 min, 4°C) and the supernatant containing secreted proteins recovered (see *2.4.4*). Cellular contents were extracted by incubating the filter papers in 0.5 M NaCl/1.5% (v/v) Triton X-100 (VWR)

containing Complete Mini protease inhibitor cocktail tablets (Roche, Basel, Switzerland)). Samples were sonicated (10 x 1 s pulses; MSE Soniprep 150; Sanyo, Leicestershire, UK), centrifuged (15,000 *g*, 5 min, 4°C) and the supernatant measured for myeloperoxidase by oxidation of 2.5 mM 4-aminoantipyrine (dissolved in phenol) in the presence of 1.7 mM H_2O_2[361]. Absorbance was measured at 510 nm with a Shimadzu UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan). Horseradish peroxidase (HRP) was used as a standard (0-200 μg/ml).

2.4.4 Enzyme-Linked Immunosorbent Assays

Commercially available ELISA kits were purchased for IL-1β, IL-8, TNF-α, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) (R&D Systems), albumin (Alpha Diagnostic International, TX, USA), histamine (IBL Hamburg, Hamburg, Germany), and C3a-desArg (Progen, Heidelberg, Germany). Assays were conducted following the instructions from the manufacturer, with recombinant human protein standards. Concentrations of these mediators were measured in 30 min or 24 h skin window fluid from *2.4.3*, with appropriate dilutions determined to obtain readouts within the linear range of the assay. Sandwich ELISAs were used for cytokines, and competitive binding ELISAs for other mediators.

Protocols for the IL-1β, IL-8, TNF-α and C3a-desArg ELISAs were similar for each kit. Briefly, samples or standards were incubated for 2 h (IL-1β, IL-8 and TNF-α) or 1 h (C3a-desArg) in monoclonal antibody-coated 96-well plates. Wells were washed and incubated with secondary antibody conjugated to HRP for 1 h. Washes were repeated, and substrate solution consisting of tetramethylbenzidine (TMB) and H_2O_2 applied for 30 min (IL-1β, IL-8 and TNF-

α) or 10 min (C3a-desArg) in the dark. The colour reaction was terminated with 1 M H_2SO_4 (VWR), and the optical density of each well determined immediately using a microplate reader (Anthos Labtec Instruments, Salzburg, Austria) set to 450 nm.

For PGE_2 and LTB_4, samples or standards were added to a 96-well plate coated with goat anti-mouse (PGE_2) or anti-rabbit (LTB_4) polyclonal antibody, with wells reserved to assess total activity (TA), non-specific binding (NSB), maximum binding (B_0) and a blank. Purified PGE_2/LTB_4 (as appropriate) conjugated to alkaline phosphatase (ALP) was added to each well excluding the TA and blank wells, followed by mouse monoclonal anti-PGE_2 or rabbit polyclonal anti-LTB_4 antibody to all wells except the NSB, TA and blank wells. These were incubated for 24 h at 4°C. Wells were washed then 5 µl of the PGE_2/LTB_4-ALP conjugate added to the TA well. Substrate solution containing p-nitrophenyl phosphate was added and incubated for 1 h at 37°C. The reaction was stopped with trisodium phosphate solution and the optical density determined at 405 nm. To normalize the results, each reading had the NSB optical density subtracted then was divided by the optical density of B_0.

For histamine, samples and standards were acylated in glass tubes using the reagents provided for 30 min. These were then diluted in 2 ml Assay Buffer and 50 µl of each added to a 96-well plate coated with goat anti-rabbit antiserum, together with 50 µl histamine conjugated to HRP and 50 µl rabbit anti-histamine antiserum. This was incubated for 3 h, washed 4 times, and the colour reaction developed with 200 µl TMB substrate solution. The reaction was stopped after 15 min with 100 µl H_2SO_4, and the optical density read at 450 nm.

For albumin, samples or standards were added to a 96-well plate coated with an anti-albumin antibody, in the presence of a secondary anti-albumin antibody conjugated to HRP. This was incubated for 30 min with gentle shaking, washed 4 times and HRP substrate solution added for 10 min in the dark. The reaction was stopped with 1 M H_2SO_4 and absorbance at 450 nm measured.

Efficiency of cytokine recovery using this technique was determined *in vitro* with filter papers blocked for 1 h in normal human serum then preincubated for 24 h with known concentrations of recombinant human IL-8. Extraction took place as above (see *2.4.3*). Recovery efficiency was 76.02 ± 3.80% (mean ± s.d., n = 6), equivalent in the 10^{-9} and 10^{-6} g/ml ranges.

2.4.5 Cytokine protein array

Membranes arrayed with 42 different cytokines (RayBio™ Human Cytokine Array III; RayBiotech, GA, USA; Table 2.2) were probed with skin window fluid, following the instructions from the manufacturer. Briefly, membranes were blocked for 30 min and exposed for 2 h to skin window fluid (diluted 1:20 in block buffer) from the first supernatant derived in *2.4.3*. Membranes were washed, incubated with biotin-conjugated antibodies against the different cytokines for 2 h, washed and incubated with HRP-conjugated streptavidin for 60 min. Washes were repeated, detection carried out using the reagent provided, and imaged using a ChemiDoc machine (Bio-Rad, CA, USA).

2.4.6 TGF-β activity assay

TGF-β activity was determined using mink lung epithelial cells (MLECs; gift from D. Rifkin) that had been transfected with an 800 bp fragment of 5' PAI-1

IL-1β	IL-1β	IFN-γ	IFN-γ	TGF-β1	TGF-β1	Pos	Pos
IL-1α	IL-1α	IL-15	IL-15	TARC	TARC	Neg	Neg
I-309	I-309	IL-13	IL-13	SDF-1	SDF-1	Leptin	Leptin
GRO-α	GRO-α	IL-12	IL-12	SCF	SCF	PDGF-β	PDGF-β
GRO	GRO	IL-10	IL-10	RANTES	RANTES	VEGF	VEGF
GM-CSF	GM-CSF	IL-8	IL-8	MIP-1δ	MIP-1δ	Tpo	Tpo
G-CSF	G-CSF	IL-7	IL-7	MIG	MIG	OSM	OSM
ENA-78	ENA-78	IL-6	IL-6	MDC	MDC	Ang	Ang
Neg	Neg	IL-5	IL-5	M-CSF	M-CSF	IGF-1	IGF-1
Neg	Neg	IL-4	IL-4	MCP-3	MCP-3	EGF	EGF
Pos	Pos	IL-3	IL-3	MCP-2	MCP-2	TNF-β	TNF-β
Pos	Pos	IL-2	IL-2	MCP-1	MCP-1	TNF-α	TNF-α

Table 2.2 Layout of multiple cytokine arrays.

fused to a luciferase reporter as previously described[362]. The cells were maintained in D-MEM (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (Invitrogen) and penicillin/streptomycin. Cells were plated to confluence. A standard curve was derived using recombinant human TGF-β (0-1 ng/ml). Skin window fluid from *2.4.3* was diluted to 3-fold, 10-fold and 20-fold, and then heat-activated at 85°C for 10 min. These were added to MLECs with an equal volume of medium and incubated for 18 h. Medium was aspirated and cells washed with phosphate buffered saline (PBS; Oxoid, ON, Canada), lysed and incubated on a rocker for 30 min. Luciferase reagents (Promega, WI, USA) were added and the signal detected using a luminometer (PerSeptive Biosystems, MA, USA).

2.4.7 Protein gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using standard Laemmli methods[363]. Mini gels of 10 x 8 x 0.08 cm were cast using 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide solution (Protogel; National Diagnostics, GA, USA), for a final concentration of 8-12% acrylamide depending on the size of the protein of interest. In all cases, a stacking gel containing 5% acrylamide was used. Samples of skin window cell extracts were prepared by adding 20% volume of sample buffer (0.06 M Tris-HCl pH 6.8, 1% sodium dodecyl sulfate (SDS), 0.03 M sucrose and 17.9 μM β-mercaptoethanol (VWR), 0.04% (w/v) bromophenol blue (VWR)). They were heated to 95°C for 2 min and spun down (15,000 *g*, 2 min, 4°C). Hoefer Mighty Small II SE 250 systems (Hoefer, CA, USA) were used for electrophoresis, operated at 20 mA per gel.

2.4.8 Western blotting

Samples were transferred from gels to nitrocellulose membrane (Hybond-P; Amersham Biosciences, Buckinghamshire, UK) using a semi-dry blotter (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad), in transfer buffer (200 mM glycine, 0.1 % SDS (w/v), 10% methanol (v/v)), 25 mM Tris-HCl pH 8.8) for 1 h at 1.25 mA/cm^2. Protein sample and transfer quality were confirmed by reversible staining with Ponceau Red. Membranes were blocked for 1 h with 5% non-fat milk (w/v) in TBS/0.05% Tween-20 (v/v). Membranes were probed with primary antibodies (1:500, in 5% milk) against myeloperoxidase or lactoferrin (produced in the laboratory by E. Reeves by immunization of rabbits with purified recombinant proteins) overnight at 4°C.

Membranes were washed (4 x 5 min) and incubated with HRP-conjugated anti-rabbit IgG antibody (Amersham Biosciences; 1:2,000 in 5% milk) for 1 h, washed again, developed using the Enhanced Chemiluminescence method (ECL PLUS; Amersham Biosciences), and the autoradiograph processed with an X-Omat film developer (Kodak, Hertfordshire, UK).

2.4.9 Multi-channel Western blots

In a separate set of experiments, cells were removed from filter papers using a cell scraper and lysed in 1 ml hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM dithiothreitol (DTT), Protease Inhibitor Cocktail, 0.5% IGEPAL and phosphatase inhibitors 25 mM NaF, 1 mM Na_3VO_4 and 25 mM $Na_4P_2O_7$). The mixture was rotated at 4°C for 10 min and then centrifuged

(15,000 *g*, 3 min). The supernatant was taken as the cytosolic fraction, and the pellet retained for nuclear proteins (see *2.4.10*).

Cytosolic protein was quantified by Bradford assay[364], by incubating with Coomassie brilliant blue G-250 dye (Pierce, IL, USA) then comparing the absorbance at 595 nm to a standard curve of bovine serum albumin (BSA; 0-200 μg/ml). Equal concentrations (250μg/ml) were run across the top of a 10% SDS-polyacrylamide gel containing a single long well. Protein was transferred to a Hybond-P membrane and blocked as before (see *2.4.8*). The membrane was then placed on a Mini-Protean II multi-screen apparatus (Bio-Rad) that allows probing with 20 different antibodies on a single blot. Primary antibodies were incubated overnight (15:2,000 in 5% BSA (w/v)/TBST; see Table 2.3 for list of primary antibodies used). The membrane was washed (5 x 5 min), incubated with donkey anti-rabbit peroxidase-conjugated secondary antibody for 1 h, washed again and positive bands visualized with ECL Plus.

To determine phosphorylation status of different PKC isoforms, samples from different subjects were run on the same gel (as in *2.4.8*), then transferred to Hybond-P membrane and probed using the same protocol with antibodies against pan-phosphorylated PKC, and phosphorylated PKCα/β, PKCδ, PKCζ/λ, PKCθ and PKCμ (Cell Signalling Technology, MA, USA).

2.4.10 DNA-binding protein arrays

Nuclear pellets were prepared as described in *2.4.9*. These were resuspended in 200 μl Complete Lysis Buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1mM EDTA, 1mM DTT, 10% glycerol (VWR) and Protease Inhibitor Cocktail) and incubated on a shaking platform at 4°C for 30 min. The extract was centrifuged

Protein	Phosphorylation Site	Company	Catalogue Number
4E-BP1	Ser65	Cell Signalling Technology	#9451
Akt	Ser473	Cell Signalling Technology	#9271
Akt	Thr308	Cell Signalling Technology	#9275
Bad	Ser112	Cell Signalling Technology	#9296
Bad	Ser155	Cell Signalling Technology	#9297
cdc2	Tyr15	Cell Signalling Technology	#9111
cdc25	Ser216	Cell Signalling Technology	#9528
Chk1	Ser345	Cell Signalling Technology	#2341
Cleaved caspase-3	np	Cell Signalling Technology	#9661
Cleaved caspase-7	np	Cell Signalling Technology	#9491
Cleaved caspase-9	np	Cell Signalling Technology	#9501
Cleaved PARP	np	Cell Signalling Technology	#9541
c-Raf	Ser338	Cell Signalling Technology	#9427
CREB	Ser133	Cell Signalling Technology	#9191
EGFR	Tyr1173	Cell Signalling Technology	#2244
eIF4E	Ser209	Cell Signalling Technology	#9741
FKHR	Ser256	Cell Signalling Technology	#9461
GSK-3β	Ser9	Cell Signalling Technology	#9336
IκB-α	Ser32	Cell Signalling Technology	#9241
Jak1	Tyr1022/1023	Cell Signalling Technology	#3331
Jak2	Tyr1007/1008	Cell Signalling Technology	#3771
MEK1/2	Ser217/221	Cell Signalling Technology	#9121
Mnk1	Thr197/202	Cell Signalling Technology	#2111
mTOR	Ser2448	Cell Signalling Technology	#2971
NF-κB p105/p50	np	Cell Signalling Technology	#3035
NF-κB p65	Ser276	Cell Signalling Technology	#3037
p38 MAP kinase	Thr180/Tyr182	Cell Signalling Technology	#9211
p44/p42 MAP kinase	Thr202/Tyr204	Cell Signalling Technology	#9101
p53	Ser15	Cell Signalling Technology	#9284
p53	Ser392	Cell Signalling Technology	#9281
p53	Ser6	Cell Signalling Technology	#9285
p70 S6 kinase	Thr389	Cell Signalling Technology	#9205
p90rsk	Ser381	Cell Signalling Technology	#9341
PDK1	Ser241	Cell Signalling Technology	#3061
PI3 kinase	Tyr508	Santa Cruz	sc-12929
PKA catalytic subunit β	Ser338	Biosource	44-992
PKA substrate		Cell Signalling Technology	#9621
PKCα/β	Thr638/641	Cell Signalling Technology	#9375
PKCδ	Ser643	Cell Signalling Technology	#9376
PKCδ	Thr505	Cell Signalling Technology	#9374
PKCμ	Ser744/748	Cell Signalling Technology	#2054
PKCμ	Ser916	Cell Signalling Technology	#2051
PKCθ	Thr538	Cell Signalling Technology	#9377
PKCζ/λ	Thr410/403	Cell Signalling Technology	#9378
PLCγ1	Tyr783	Cell Signalling Technology	#2821
PLCγ2	Tyr1217	Cell Signalling Technology	#3871
PTEN	Ser380	Cell Signalling Technology	#9551
Raf	Ser259	Cell Signalling Technology	#9421
Rb	Ser780	Cell Signalling Technology	#9307
Rb	Ser795	Cell Signalling Technology	#9301
Rb	Ser807/811	Cell Signalling Technology	#9308
S6 ribosomal protein	Ser235/236	Cell Signalling Technology	#2211
SAPK/JNK	Thr183/Tyr185	Cell Signalling Technology	#9251
Src	Tyr416	Cell Signalling Technology	#2101
Src	Tyr527	Cell Signalling Technology	#2105
Syk	Tyr323	Cell Signalling Technology	#2715
Syk	Tyr525/526	Cell Signalling Technology	#2711
TrkA	Tyr490	Cell Signalling Technology	#9141
Zap-70	Tyr319	Cell Signalling Technology	#2701

Table 2.3 Antibodies used for multi-channel Western blots.

(15,000 *g*, 10 min, 4°C) and the supernatant used to probe TranSignal Protein/DNA Arrays Version I (Panomics, CA, USA) following the instructions from the manufacturer. These arrays are spotted with 54 different consensus-binding sequences, each corresponding to a specific transcription factor. In brief, 15 μg of total nuclear proteins were incubated with biotin-labeled DNA-binding probes (Tran-Signal Probe Mix) to allow the formation of protein-DNA (or TF-DNA) complexes. These were separated from free probes by electrophoresis in agarose gels. Probes present in the complexes were eluted and hybridized to the TranSignal membrane dotted with complementary non-labeled probes, and signals detected by chemiluminescence.

2.4.11 Reverse transcriptase-Polymerase Chain Reaction

Cells attached to approximately one-eighth of the filter paper were lysed by vortexing in guanidium thiocynate solution (RLT buffer; Qiagen) supplemented with 0.1 M 2-mercaptoethanol. Total RNA was isolated with the RNeasy mini kit (Qiagen), 1 μg of which was reverse transcribed by use of dT primers. A cDNA equivalent corresponding to 20 ng of total RNA was amplified in each reaction. The primers used for PCR were:

CD14 primer (forward): 5'-CACGCCAGAACCTTGTGAG-3'

CD14 primer (reverse): 5'-CCCAGTCCAGGATTGTCAG-3'

GAPDH primer (forward): 5'-GTCACCAGGGCTGCTTTTAAC-3'

GADPH primer (reverse): 5'-TGCTTCACCACCTTCTTGATG-3'

As a control of mRNA input, GAPDH mRNA levels were determined for each sample in separate reactions. The PCR reactions contained dNTPs and buffer as supplied by the manufacturer, 500 pM of each specific primer and 3 U Taq polymerase (RedTaq). Transcripts were amplified for 29 cycles with CD14 primers (30 s at 94°C, 30 s at 56°C, 30 s at 72°C) and 20 cycles for GAPDH primers (30 s at 94°C, 30 s at 58°C and 45s at 72°C), followed by 7 min at 72°C. PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination. For GAPDH amplification, PCR was performed with different cycle numbers to ensure that amplification was occurring within the linear range.

2.4.12 Analysis

Comparisons within one group of subjects for a treatment effect were performed using a 2-tailed students' t-test (Microsoft® Excel 2003). For those between different groups and under different conditions, data were compared using a 2-way ANOVA with Bonferroni post-tests (Graphpad Software). The threshold for significance was $P < 0.05$.

2.5 Macrophage cytokine production

2.5.1 Macrophage isolation and culture

Peripheral venous blood was collected into 5 U/ml heparin (CP Pharmaceuticals, Wrexham, UK) and mixed with an equal volume of balanced salt solution (0.14 M NaCl, 0.01% anhydrous D-glucose, 5 μM $CaCl_2$, 98 μM $MgCl_2$, 0.54 mM KCl, 14.5 mM Tris HCl pH 7.6). Mononuclear cells were isolated by differential centrifugation (15 min, 800 *g*, 20°C) over Ficoll-Paque PLUS (Amersham

Biosciences) and washed repeatedly with phosphate-buffered saline (PBS) using low speed spins (15min, 200 *g*) to remove platelets. Cells were resuspended in medium and plated at a density of approximately 5 x 10^6 cells/ml in 8 cm^2 tissue culture dishes (VWR). The medium used was RPMI-1640 (Invitrogen) supplemented with 10% normal human serum and penicillin/streptomycin for the *Escherichia coli* LPS (see *5.2.1*) and MDP (see *5.2.2-5.2.4*) experiments, and X-Vivo-15 (Cambrex, MD, USA) containing gentamicin for the other experiments. Cells were cultured for 5 days at 37°C, 5% CO_2.

Macrophage culture purity was assessed by immunostaining for the pan-macrophage marker CD68[365] (Santa Cruz Biotechnology, CA, USA). Cells were fixed on cover slips in 4% paraformaldehyde for 10 min, washed 3 times with PBS and blocked with normal goat serum (1:1,000 in TBST/10 mM $NaBH_4$) for 1 h. This was replaced with primary antibody at a dilution (1:200) overnight at 4°C, and then washed four times with PBS. Secondary antibody was donkey anti-mouse conjugated to FITC (1:200 in TBST) and incubated for 1 h in the dark. Slides were washed then mounted with VectaShield anti-fade mountant containing DAPI nuclear stain (Vector Laboratories, CA, USA). For controls, primary antibody was omitted or lymphocytes were used; neither showed any staining. The numbers of positive cells in five randomly selected high power fields per cover slip were averaged and calculated as a percentage of the positive staining nuclei. In all samples tested, purity was >95%.

2.5.2 LPS stimulation and IL-12 ELISA

Macrophages were incubated for 15 h at 37°C and 5% CO_2 with or without 100 ng/ml *Escherichia coli 0157:B8* LPS. The supernatant was centrifuged (15,000 *g*,

2 min) then assayed for IL-12p70 by ELISA; the protocol for the latter was similar to the sandwich ELISAs used previously (see *2.4.4*).

2.5.3 MDP stimulation and RNA isolation

Macrophages were incubated for 15 h with or without 100 ng/ml MDP. This concentration was reported to cause differential effects on cells carrying wild type and variant *CARD15* alleles respectively at the time these experiments were performed[288]. Cells were lysed in TRIzol reagent (Invitrogen) and total RNA isolated by extraction with chloroform (VWR) followed by precipitation with isopropanol (VWR). The pellet was washed with 75% ethanol (VWR), air-dried, and re-dissolved in DEPC-treated water. The quality and quantity of these samples was determined using an Agilent Bioanalyzer (Agilent Technologies, CA, USA) following the instructions from the manufacturer. In the event that insufficient RNA was isolated, equal amounts of sample from subjects were combined to produce adequate starting material. These were then treated as a new individual sample. Expression patterns from samples pooled in this way were completely consistent with results obtained from individuals.

2.5.4 DNA microarrays

Approximately 5 μg total RNA from each independent sample was processed to produce biotinylated cRNA targets. Double-stranded cDNA was synthesized by reverse transcription of twice-purified mRNA using T7-(T_{24}) primers (Genset, CA, USA) and the Superscript II cDNA Synthesis System (Invitrogen). The cDNA was used as a template for the generation of biotin-labeled *in vitro* transcription products using the Ambion T7 Megascript System (Ambion) and

biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics Inc., NY, USA). The biotinylated cRNA products were prepared and hybridized to Human Genome U133A arrays following standard Affymetrix procedures (www.affymetrix.com) using an Affymetrix Fluidics station (Affymetrix, CA, USA) and, after extensive washing, were scanned on a GeneArray scanner G2500A (Hewlett Packard, CA, USA).

Each array demonstrated control parameters within recommended limits (Raw Q ≤30, background ≤100, GAPDH 3':5' ratio <4). Global normalization was performed on each chip and data scaled against a reference chip. After exclusion of the highest and lowest 2% of the data points from each chip, the mean and standard deviation of the $\log_2$(signal) from each chip were calculated and a mean-corrected $\log_2$ value produced for each data point. The scale factor for the chips was calculated as the ratio of mean-corrected $\log_2$ values of the reference [R] and each target chip [T] as:

$$\Sigma[\log_2(\text{signal}_R) - \text{mean } \log_2(\text{signal}_R)] \,/\, \Sigma[\log_2(\text{signal}_T) - \text{mean } \log_2(\text{signal}_T)]$$

2.5.5 Gene expression analysis

Data from *2.5.4* were analyzed using the commercially available Spotfire® visualization and analysis program (www.spotfire.com; Spotfire, MA, USA). Probes were filtered for those showing at least 2-fold change in expression, sorted using a Euclidean distance matrix and clustered hierarchically then joined to form a dendrogram. The full length genes were annotated using the in-house system developed by Inpharmatica, which relies on the Biopendium™ (Inpharmatica, London, UK). All genes in which there was a change in the mean level of expression of 2-fold or greater were analyzed for the effects of treatment

between the control and patient groups by 2-way ANOVA with Bonferroni post-tests (Graphpad Software).

2.5.6 Post-genomic verification

Post-genomic verification was provided by measuring cytokine secretion in cell culture supernatants from unstimulated and MDP-stimulated cells from *2.5.3*. Samples were assayed using ELISAs for IL-1β, IL-8, IL-10, IL-12 and TNF-α (R&D Systems), following the protocol described in *2.4.4*. Protein from macrophages was also prepared by lysis in sample buffer containing protease inhibitors as described previously (see *2.4.7*) and a Western blot performed (see *2.4.8*) using a rabbit anti-TIMP-3 antibody (1:1,000 in 5% milk/TBST).

2.5.7 Structural modelling

The full length protein sequence of CARD15 from SwissProt (Q9HC29; Swiss Institute of Bioinformatics, Geneva, Switzerland) was annotated using PSI-BLAST[366] and GenomeThreader[367] fold recognition algorithms as implemented in the Biopendium™ [368]. To better define domain boundaries, 3D structures and aid alignments, the secondary structure of the LRR domain was predicted using the PSI-PRED algorithm[368] obtained from the Biopendium™; if necessary, some manual adjustments were made. Alignments were used to create homology models using MODELLER[369] v6.1 (University of California, CA, USA). The molecular surface and electrostatic potentials of the models were calculated using GRASP[370] (University of Leeds, Leeds, UK).

2.5.8 Collection of wound fluid

Wound fluid was collected from otherwise healthy patients undergoing elective surgery for inguinal hernia repair. Swabs, used to remove blood and fluid from the wound, were retrieved 2 min after the incision and the fluid eluted into 10 ml sterile normal saline. This was placed immediately on ice, centrifuged (20,000 *g*, 10 min, 4°C), snap frozen and stored at -70°C until use. Serum was derived from peripheral venous blood collected into a serum separator tube (Becton Dickinson), and then spun (2,000 *g*, 10 min).

2.5.9 2D gel electrophoresis and mass spectrometry

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates proteins first according to pI (through isoelectric focusing) followed by molecular weight (by SDS-PAGE). Protein in each sample was quantified using the Bradford assay. Protein was precipitated with 110 µl/ml trichloroacetic acid in acetone (VWR), spun down and washed in 80% acetone (v/v), dried and resuspended in CHAPS buffer (8 M urea, 2 M thiourea, 4% CHAPS, 0.8% (w/v) ampholyte, 1% (v/v) Triton X-100, 65 mM DTT, Complete Mini Protease inhibitors). The sample was heated to 95°C for 5 min, sonicated (10 x 10 sec pulses) then added to immobilized pH gradient strips (pH 3-10; Amersham Biosciences). This was overlaid with 500 µl mineral oil, then run overnight on an IGPhor machine (Amersham Biosciences) for 60 kVh. Strips were equilibrated twice for 15 min each in equilibration buffer (30% glycerol (v/v), 2% SDS (w/v), 6 M urea, 50 mM Tris-HCl pH 6.8). The first equilibration step contained 2%

(w/v) DTT and the second 4.5% (w/v) iodoacetamide. The second dimension was run on a large 10% SDS-PAGE gel (see *2.4.7*) at 40 V overnight.

Gels were fixed in 50% (v/v) methanol, 5% (v/v) acetic acid (VWR) for 20 min. They were then washed in 50% methanol for 10 min and H_2O for 2 h, sensitized in 0.02% (w/v) $Na_2S_2O_3$ for 1 min, washed twice with H_2O, then incubated in cold 0.1% (w/v) $AgNO_3$ for 20 min. Gels were washed with H_2O and developed in 0.04% (v/v) formalin/2% (w/v) Na_2CO_3. Staining was terminated with 5% (v/v) acetic acid[371]. Spots of interest on the gel were cut and soaked in 200 µl 50 mM NH_4HCO_3 for 15 min. The buffer was removed and gel pieces dried in 200 µl acetonitrile for 10 min then in a Speedvac (Thermo) for 30 min followed by rehydration in 10 mM DTT/100 mM NH_4HCO_3 for 30 min at 56°C; the acetonitrile step was then repeated. Samples were digested with 75 ng/µl trypsin/25 mM NH_4HCO_3 and incubated overnight at 30°C. The sample was spotted onto a target plate and covered with 10 mg/ml α-cyano-4-hydroxycinnamic acid matrix in 35% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and analyzed by MALDI-TOF in the laboratory of J. Godovac-Zimmermann.

2.5.10 IL-8 ELISA development kit

Macrophages from buffy coats were cultured as described previously (see *2.5.1*) and incubated with various dilutions of wound fluid. IL-8 secretion was measured using an ELISA Development Kit (R&D Systems). The anti-IL-8 capture antibody was used to coat a 96-well plate overnight. This was washed three times with Wash Buffer (0.05% Tween-20 in TBS), then blocked with 1% BSA/0.05% NaN_3 in TBS, washed, then samples and standards added for 2 h.

Wells were washed again then streptavidin-HRP added for 20 min in the dark. The colour reaction was developed using TMB substrate solution for 20 min in the dark, stopped with 1 M H_2SO_4 and read at 450 nm.

2.5.11 Wound fluid inhibitor studies

The effects of different inhibitors on wound fluid-induced IL-8 secretion were assessed. These included pre-treatment of the wound fluid with H-d-Phe-Pro-Arg-CH_2Cl (PPACK, 10 nM pre-heated to 37°C), Complete Mini Protease inhibitors (1 tablet/10ml) or heating to 56°C for 30 min, or pre-treatment of the macrophages for 30 min prior to addition of wound fluid with 100 ng/ml cholera toxin (Calbiochem, CA, USA) or pertussis toxin (Calbiochem). Individual inhibitors were incubated with the cells in the absence of wound fluid to confirm no adverse effects on viability.

2.5.12 CH_{50} assay

Antibody-sensitized sheep erythrocytes were suspended in ice-cold gelatin veronal buffer (GVB^{2+}). A 200 µl aliquot was lysed in 2.8 ml dH_2O and the optical density read at 405 nm (OD_{405}). Further GVB^{2+} was added to give a concentration of 10^8 cells/ml (final volume = [OD_{405}/0.73] x 10 ml). Serum was prepared from 3 healthy controls and 200 µl of this or wound fluid added to 50 µl erythrocytes. Controls were GVB^{2+} or dH_2O for 0% and 100% lysis respectively. Samples were incubated at 37°C for 30 min (the reaction was then stopped with 250 µl ice-cold PBS), spun (2,000 *g*, 5 min, 4°C) and the OD_{405} measured. CH_{50}

was calculated as the titre of serum or wound fluid producing the mean of the OD_{405} for GVB^{2+} and dH_2O[372].

2.5.13 Response to alternative inflammatory agonists

Macrophages were cultured for 5 days in X-Vivo-15 medium (serum-free), then re-plated at equal densities in a 96-well plate and allowed to adhere overnight. These were stimulated for 6 h with wound fluid (300 μg/ml protein), C5a (500 ng/ml), TNF-α (5 ng/ml; Calbiochem) or *Salmonella typhimurium* LPS (100 ng/ml). The supernatants were removed and measured for the production of IL-8 by ELISA as described in *2.5.10*. Numbers of viable cells in each well were determined using the Cell Counting Kit-8 (Alexis, CA, USA)[373]. Briefly, 100 μl medium and 8 μl 5 mM WST-8 were added to the cells, which were incubated for 90 min at 37°C, 5% CO_2, and the A_{450} measured. This assay was validated by measuring the A_{450} for serial dilutions of buffy coat macrophages. Subjects from different groups were stimulated and compared simultaneously. Basal cytokine secretion was minimal and similar in all groups. Cytokine secretion was expressed normalized to cell count with basal secretion subtracted.

2.5.14 Phosphorylation of p44/p42 MAP kinase

Macrophages were prepared as above (see *2.5.13*) and stimulated for 2 min with 500 ng/ml C5a, then washed in ice-cold PBS and lysed in sample buffer containing protease and phosphotase inhibitors (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 tablet Complete Mini Protease inhibitors per 10 ml, 0.05% Triton X-100, 0.5% (w/v) SDS, 0.5% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.02% bromophenol blue). These were

run on a 10% SDS-polyacrylamide gel (see *2.4.7*) then blotted (see *2.4.8*) against rabbit anti-phosphorylated p44/p42 MAP kinase (1:1,000 in 5% BSA/TBST) for 1 h. The secondary antibody was donkey anti-rabbit conjugated to HRP (1:2,000 in 5% milk/TBST).

Membranes were then stripped (62 mM Tris-HCl pH 6.8, 0.7% (v/v) 2-mercaptoethanol, 2% (w/v) SDS) for 1 h at 50°C on a rotator, washed, blocked and re-probed with rabbit anti-p44/p42 MAP kinase (Cell Signalling Technology; 1:1,500 in 5% BSA/TBST) for 1 h. The same secondary antibody was used (1:3,000).

2.5.15 Analysis

For single comparisons, the 2-tailed students' t-test was used (Microsoft® Excel 2003). Multiple comparisons were made by 1-way Kruskal-Wallis ANOVA with Dunn post-tests or 2-way ANOVA with Bonferroni post-tests as appropriate (Graphpad Software). The threshold for significance was $P < 0.05$.

2.6 Bacterial injections

2.6.1 Bacterial culture and sterilisation

A fully antibiotic-sensitive clinical isolate of *Escherichia coli* NCTC 10418 was obtained from UCLH Clinical Microbiology and grown overnight in minimal citrate medium (5 g/l glucose, 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1 g/l ammonium acetate, 2.5 g/l sodium citrate, 0.2 g/l $MgSO_4$, 10 mg/l ferric ammonium citrate, 10 mg/l $MnCl_2$, 20 mg/l $CaCl_2$) supplemented with 1.25 g/l yeast extract (Oxoid) in sterile conditions. The bacteria were spun down, washed and resuspended in PBS and killed by heating to 80°C for 30 min. They were

washed once in sterile PBS, aliquoted, and spun down; the pellet was snap frozen and stored at -70°C. Sterility was confirmed by culture. Bacterial concentrations were determined by measuring the optical density of the sample at 595 nm (OD for 1 x 10^8 bacteria/ml = 0.365 for *Escherichia coli*[374]). Bradford assays were performed to determine the amount of protein present (see *2.4.9*).

2.6.2 Labelling with ^{99m}Technetium

Bacteria were incubated with for 5 min with 30 MBq [^{99m}Tc]-sodium pertechnetate (Amersham Biosciences), reconstituted in the presence of either 40 μM stannous chloride[375] (reducing the chemically stable species to a reactive ligand) or lipophilic exametazime (Amersham Biosciences). Both led to incorporation of the label into the cell membrane. Following this, 0.5 ml of 0.9% NaCl (injection grade) was added and the mixture centrifuged (15,000 *g*, 3 min). The supernatant was removed and the bacteria washed with 0.9% NaCl, centrifuged then re-suspended in 1 ml NaCl. Radioactivity was measured to calculate labeling efficiency.

The stability of the label was assessed following 6 h incubation with normal human serum (see *2.5.8*) or neutrophils. Neutrophils were obtained from peripheral venous blood collected under heparin. Red cells were removed by sedimentation with dextran (1% final concentration (w/v); mean MW = 260 kDa) and granulocytes isolated by centrifugation through Lymphoprep[376]. Further erythrocytes in the neutrophil pellet were lysed by exposure to hypotonic dH_2O, immediately followed by an equal volume of 1.8% (w/v) NaCl. Neutrophils were resuspended in Dulbecco's PBS containing 5 mM glucose. Radioactivity was measured with a handheld γ-counter.

2.6.3 Bacterial injections

Bacteria for injection were thawed and resuspended at a protein concentration of 10 mg/ml in injection-grade normal saline. An aliquot of this suspension (100 µl containing 1 mg or 10^9 organisms) was then injected into the volar aspect of each forearm. In the one subject into whom radiolabeled bacteria were injected, the radioactivity remaining the forearm, original sample or sample of peripheral venous blood was measured using a γ-counter and imaged using a γ-camera in the Institute for Nuclear Medicine, UCLH.

Full clinical details were documented for each subject. In addition to the investigations described below, the use of any medication over the course of the experiment was noted. Pain scores for the site of injection were taken at each time point, using a visual analog scale between 0 (no pain) and 10 (worst pain imaginable). Any other symptoms occurring over the time course of the study were recorded, as was the time of maximum symptoms and time to complete resolution. Any discomfort at the injection site that was great enough to interfere significantly with normal daily activities was treated with analgesics, usually 1 g paracetamol orally. Subcutaneous masses that developed at the injection site were measured in all three dimensions by standard B-mode ultrasound, using the settings for clinical study of soft tissue masses, in the Department of Imaging, UCLH.

2.6.4 Determination of blood flow

The area of erythema at each injection site was measured at 8 h, 24 h and 48 h following injection. The blood flow in this region was determined at the same

time points by laser Doppler imaging (MoorLDI2, Moor Instruments Ltd., Devonshire, UK)[377]. The laser was operated at a height of 30 cm from the skin surface with the arm in supination. Scans were always taken on the same default settings from the manufacturer, except for those studies in which subjects received an arterial cannula (see *2.6.9*). In the latter, the arm needed to be raised onto a cushion, such that the distance to laser, area scanned and resolution settings had to be altered (although they were consistent throughout each study). These data were only used to compare the effects of different pharmacological agents on forearm blood flow and were not included in the comparisons to other subjects or other time points, for which separate scans using the regular settings were acquired. Data were analyzed using the software provided by the manufacturer, and blood flow calculated as a volume under the curve by multiplying the area of increased flow by the mean pixel intensity for that region. In some subjects, the lower limb was also scanned as a control vascular bed, to test whether any differences in blood flow represented generalized or specific vasodilatatory responses.

2.6.5 Systemic acute phase response

Blood was collected prior to and at 24 h and 48 h following inoculation. Full blood counts were performed through the clinical service at the UCLH Haematology Department to determine circulating total white cell counts and differential, haemoglobin and platelet numbers. Serum was isolated as described previously (see *2.5.8*) and measured for concentrations of CRP and serum amyloid A (SAA) by automated microparticle capture enzyme immunoassay[378-380]. Briefly, the assay was based on two monoclonal mouse anti-CRP or SAA

antibodies, one immobilized onto microparticles, the other conjugated to alkaline phosphatase (ALP). The amount of acute phase protein captured from the sample was linearly related to the colour reaction, and compared to standards of purified CRP or SAA.

2.6.6 Multiple cytokine assay

Serum IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ were quantified using the Bio-Plex Cytokine Assay (Bio-Rad), following the manufacturer's instructions. The array system uses a combination of fluorescently-dyed microspheres (each containing two dyes in different proportions to allow identification) to which antibodies recognizing specific cytokines are bound. The antibody-coupled beads were incubated with samples or standards for 30 min in a shaker to allow binding of cytokines, then washed three times to remove unbound protein. A biotinylated detection antibody specific for a different epitope on the cytokine was added, incubated for 30 min then washed. Streptavidin-phycoerythrin was added for 10 min and a final wash performed. The constituents of each well were drawn into the Bio-Plex suspension array system, which identifies and quantitates each specific reaction based on bead colour and fluorescence, and compared to cytokine standards.

2.6.7 Bacterial digestion by neutrophils and organ bath studies

Neutrophils were isolated from three healthy controls, as described previously (see *2.6.2*). Another sample of bacteria from the same batch of *Escherichia coli* that had been administered to subjects (containing 10^9 bacteria) was opsonized with either 1 mg purified human IgG or 1 ml human serum (see *2.5.8*) for 30 min

at 37°C. These were washed 3 times in sterile normal saline, resuspended in 200 µl normal saline, added to 10^8 neutrophils and incubated for 60 min at 37°C with constant stirring. The sample was spun (13,000 *g*, 2 min) and the supernatant added immediately to superior mesenteric arteries in organ baths, where they were incubated for 1 h at 37°C.

Rings of superior mesenteric artery were obtained from male Sprague Dawley rats (200-300 g). The superior portion of the artery was dissected from its origin at the aorta to the distal portion. The vessel was placed in sterile Hanks Balanced Salt solution (HBSS), cleaned and cut into rings 3-4 mm in length. Intact rings of artery were suspended between two tungsten wires in a jacketed organ bath (25 ml volume) containing physiological salt solution, maintained at 37°C, 95% O_2, 5% CO_2. One of the wires was fixed to an external platform and the other connected to an isometric force displacement transducer (FT-03; Glass Instruments, Astro-Med UK, Slough, UK) for measurement of tension. Tension was recorded using Powerlab Chart version 4 (ADInstruments, Oxfordshire, UK). Parallel baths were used simultaneously in these experiments. Rings were maintained at a resting tension of 1 g for 1 h, following which 1 µM phenylephrine was used to contract the vessel then 5 µM acetylcholine to cause relaxation, demonstrating the presence of functional tissue with intact endothelium. This was washed out and tissues allowed to return to resting conditions before adding supernatants from the neutrophils that had digested bacteria.

The medium in the organ baths was then washed out, and the dose-response relationship between vessel mural tension and concentrations of phenylephrine (1-10,000 nM) determined. EC_{50} was calculated as the

concentration causing 50% of the maximum contraction. In all experiments, the effects of supernatants were compared to vessels that had not been pre-treated in this way, and expressed as a percentage of these controls from the same vessel at the same time.

2.6.8 LPS assay

To measure LPS liberated into the blood stream, serum from subjects taken 24 h after bacterial injection was tested using the Pyrogent-5000 assay (Cambrex). Gram-negative bacterial LPS catalyzes the activation of a pro-enzyme coagulase in lysates of amoebocytes from the *Limulus polyphemus* horseshoe crab[381]. The coagulase acts on a clotting protein, coagulogen, also present in the lysate, which then self-associates to form a gelatinous clot. The readout relies on turbidity of the reaction mix, measured as the optical density, which precedes clot formation. The samples were prepared and the assay conducted according to the instructions supplied by the manufacturer.

2.6.9 Pharmacological manipulation of blood flow

Three healthy subjects were studied 24 h following injection of bacteria to determine the effects on blood flow of intra-arterial norepinephrine (Clinalfa AG, Laeufelfingen, Switzerland; 240 pmol/min) followed by NG-monomethyl-L-arginine acetate (l-NMMA; Clinalfa AG; 4 μmol/min), with a normal saline washout between the two[382]. These studies were carried out with the subject supine in a quiet, temperature-controlled laboratory. The brachial artery of the non-dominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works Ltd., Birmingham, UK) inserted under local anaesthesia (2 ml of 1%

lidocaine). Resting blood flow was allowed to normalise following needle insertion prior to the infusion of vasoactive agents. Drugs or saline were infused continuously at 0.5 ml/min for 15 min each, and Doppler measurements made at 2 min, 5 min, 10 min and 15 min after beginning the infusion of each drug.

Some subjects were treated with 50 mg sildenafil citrate (Pfizer, NY, USA) orally at either 24 h or 48 h (2 patients) following inoculation, and the perfusion monitored at 30 min intervals over the next 90 min. Subjects had fasted for at least 8 h prior to these experiments.

2.6.10 Analysis

Comparisons within one group of subjects for a treatment effect were performed using a 2-tailed students' t-test (Microsoft® Excel 2003). For those between different groups and/or at different time points, data were compared using a 1-way or 2-way ANOVA with Dunn or Bonferroni post-tests (Graphpad Software) as appropriate. The threshold for significance was $P < 0.05$.

Chapter 3: Serial Intestinal Biopsies

3.1 Introduction

Numerous theories as to the underlying cause of Crohn's disease have been proposed[16]. The vast majority identify an over-activation of the immune response as the initiating event, either in reaction to abnormal infectious[144,383] (see *1.3*) or particulate material within the bowel[384], or as an autoimmune phenomenon[385]. Conversely, it has been proposed that the underlying abnormality relates to a failure of acute inflammation. Reduced neutrophil accumulation has been demonstrated following trauma to the skin, both in the skin window[232] and cantharidin blister[386] models. The mechanism behind this impairment was never fully elucidated.

The question remains whether the same defect applies to the bowel, the site of predilection for Crohn's lesions and therefore the most relevant if this truly represents the underlying pathogenic mechanism. In order to address this problem, we sought to develop a technique to allow examination of the acute inflammatory response in a similar manner in the bowel. No method previously existed for such an experiment. Those applicable to the skin include creating abrasive lesions[387] and raising blisters either through use of suction[388] or chemical agents[389]. Transposed to the gastrointestinal tract, these could include abrading the bowel (for example with a cytology brush) or provoking blister formation (using a suction device or, in theory, chemical infiltration of the mucosa). The viability of a blister-based method is somewhat dubious at this site,

given the fragility of the mucosa in comparison to the skin. In contrast, it is more amenable to biopsy, which should provide the required inflammatory stimulus.

3.2 Results

3.2.1 Subject characteristics

Over the course of the whole set of investigations presented in this thesis, 232 Crohn's patients were studied, along with 116 healthy controls and 105 patients with ulcerative colitis. Appendix 1 contains a full analysis of their clinical characteristics and carriage of *CARD15* polymorphisms. Of note, 14 patients with CGD were also genotyped; in this small sample carriage of *CARD15* polymorphisms showed a putative association with the development of intestinal inflammation (see *A1.3*).

Characteristics of the subjects participating in the serial biopsy study are detailed (Appendix 1: Tables A1.1-A1.3). All Crohn's patients were in complete remission, and all except one was off treatment for their inflammatory bowel disease. The latter had an ileorectal anastomosis and quiescent disease, and was willing to participate in the study. Since these individuals were very rare, this subject was included in the study despite use of mesalazine. All subjects had systemic markers of inflammation within the normal range, and none was clinically or biochemically malnourished.

3.2.2 Biopsy lesions can be easily visualized and targeted

Initial biopsies were taken under direct sigmoidoscopic vision, in the presence of two investigators. The length of insertion of the sigmoidoscope was noted, and a photograph taken of the mucosa. Biopsy induced immediate bleeding at the site

of trauma, with subsequent clot formation. At the second, post-traumatic sigmoidoscopy, the site of the earlier trauma was readily identified by the presence of the clot (Fig. 3.1A). Correct location was confirmed by the length of insertion of the sigmoidoscope and comparison of the mucosal vascular anatomy to the previous photograph. Forceps were positioned directly above the lesion (Fig. 3.1B), a second biopsy taken (Fig. 3.1C) and accurate sampling confirmed visually (Fig. 3.1D). The site of previous trauma induced by biopsy could be readily identified in all subjects. In contrast, alternative methods to traumatize the mucosa (including abrasion using a cytology brush, suction, or crushing using biopsy forceps to injure but not sample tissue), did not generate a lesion recognizable after 6 h.

3.2.3 Biopsy triggers a reproducible inflammatory response

In some control subjects, two biopsies were taken simultaneously from different sites (rectum and ileum, or two sites in the rectum). Both were re-biopsied 6 h after trauma. The correlation between the responses (number of positive-staining cells per high power field) in these duplicate biopsies was analyzed to determine variability of the technique.

There was no staining in negative control sections. No neutrophils were seen in any baseline samples (Fig. 3.2A,B), but substantial influx was observed at both sites on repeat biopsies (Fig. 3.2C-F). In familial adenomatous polyposis controls and Crohn's patients in whom 5 paired biopsies were taken from the rectum and ileum following trauma, numbers of myeloperoxidase-positive or IL-8-positive cells were highly correlated ($R^2 = 0.927$, $P < 0.05$ and $R^2 = 0.920$, $P < 0.05$ respectively). Similarly, in healthy controls in whom 4 paired biopsies were

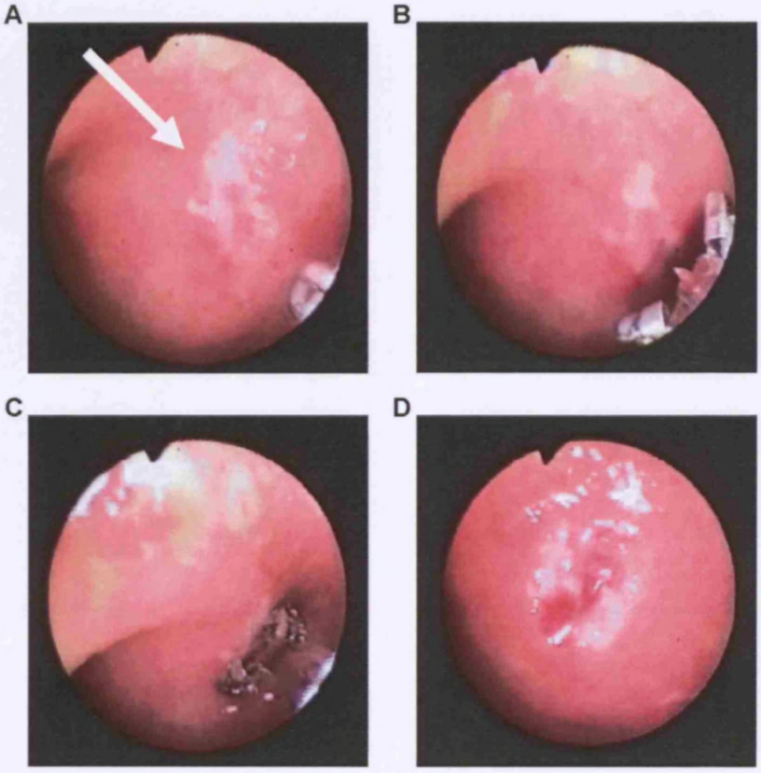


Figure 3.1 Post-traumatic biopsy in a control subject at repeat sigmoidoscopy. **(A)** The initial biopsy site was always readily identified (arrow). **(B)** Biopsy forceps were positioned directly over the lesion and **(C)** the underlying tissue sampled. **(D)** Correct siting of the second biopsy was always confirmed visually.

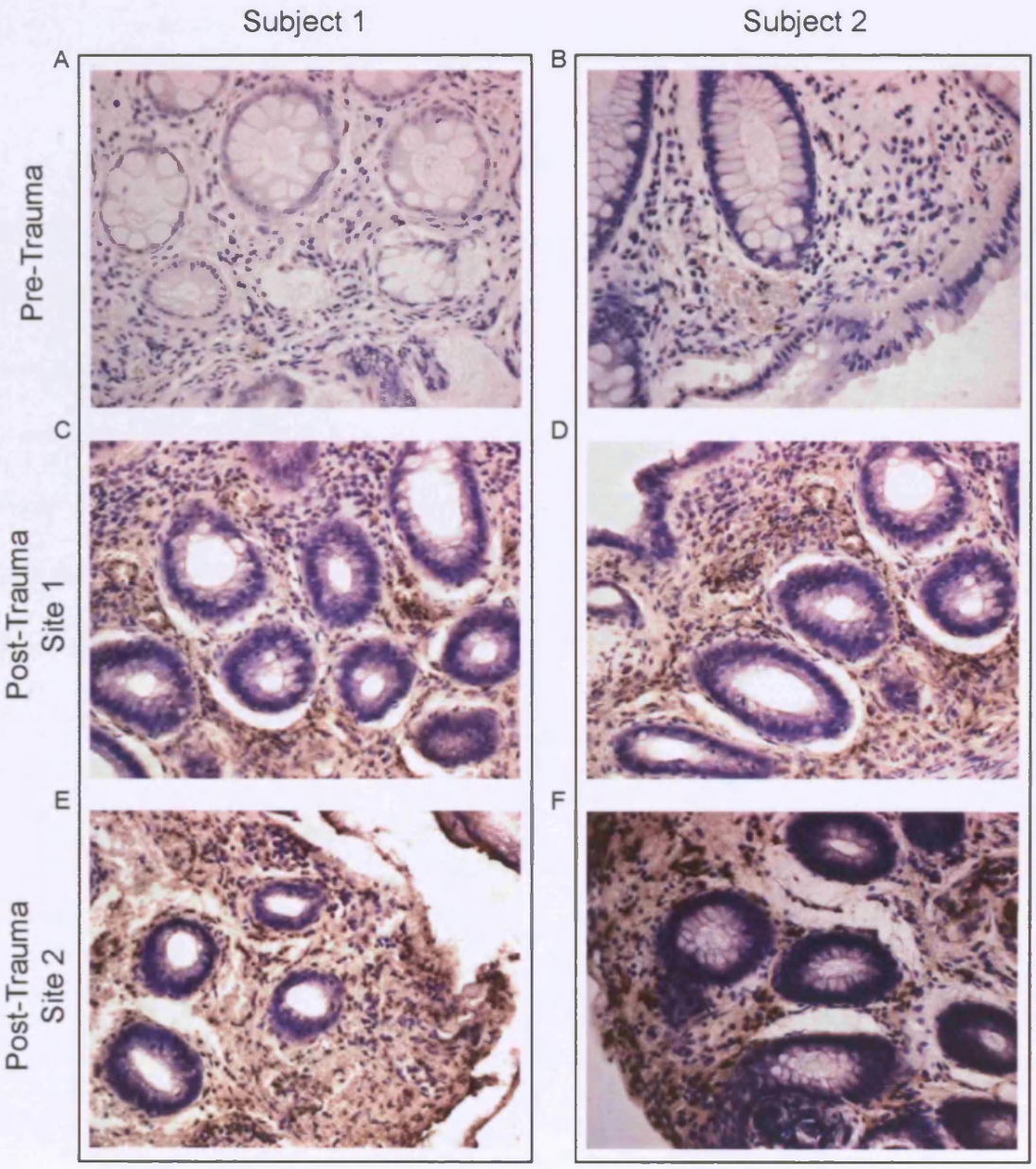


Figure 3.2 Intra-individual reproducibility of the inflammatory response induced by mucosal biopsy. Two adjacent sites in the rectum were biopsied simultaneously, in two non-inflammatory bowel disease controls. Sections were stained with an antibody against myeloperoxidase. **(A,B)** No inflammation was seen at baseline. **(C-E)** Substantial neutrophil influx induced by biopsy was observed in both controls, to a similar degree at each parallel site **(C&E** and **D&F** respectively).

taken simultaneously at two different sites in the rectum, numbers of myeloperoxidase-positive or IL-8-positive cells were highly correlated (R^2 = 0.949, $P < 0.05$ and $R^2 = 0.984$, $P < 0.01$ respectively).

3.2.4 Impaired neutrophil influx and cytokine production in Crohn's disease

In the series of Crohn's patients and non-inflammatory bowel disease controls studied, there was no inflammation in untraumatised bowel (Fig. 3.3A-D). In control subjects, trauma led to a large increase of neutrophils ($P < 0.001$) and IL-8-positive cells ($P < 0.001$) in the mucosa (Fig. 3.3E,G and 3.4A,B); the latter were predominantly interstitial macrophages and infiltrating neutrophils. Numbers of both cell types were considerably reduced in Crohn's disease (Fig. 3.3F,H and 3.4A,B). Of these patients, 4 were compound heterozygous/homozygous for polymorphisms in *CARD15* and 5 were wild type. No differences were observed between these two groups (homozygous/compound heterozygote: 38.54 ± 25.80 and 35.80 ± 13.19; wild type: 62.00 ± 8.22 and 53.53 ± 6.81; mean ± SEM for MPO and IL-8 respectively).

In addition, serial biopsies were conducted on 3 Crohn's patients and 2 control subjects whose colon had been removed, resulting in an ileorectal anastomosis. These successfully demonstrated that similar differences between the two groups were observed following trauma to the ileum (Fig. 3.4A,B).

Serial biopsies were also taken from 3 patients with ulcerative colitis. These revealed modestly elevated numbers of neutrophils in the bowel in the resting state, despite macroscopically normal mucosa and no clinical or

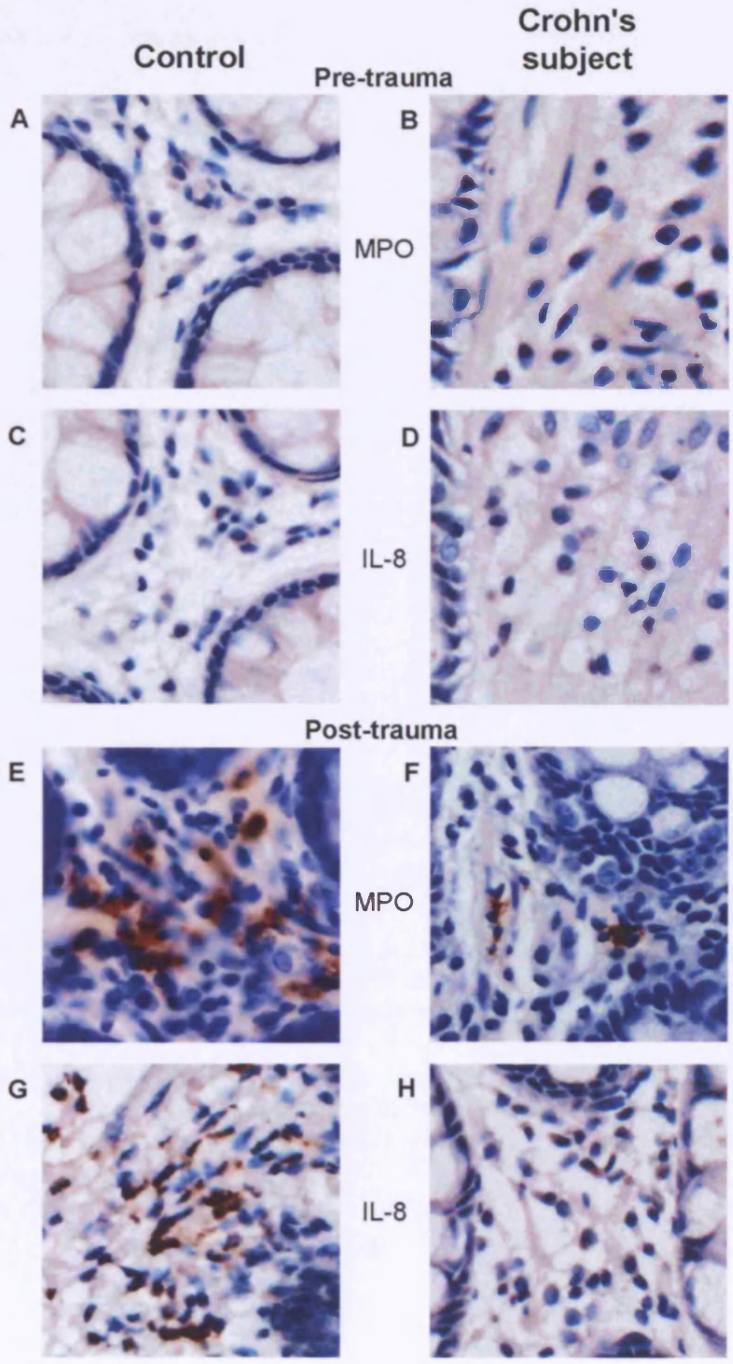


Figure 3.3 Neutrophil accumulation and IL-8 production in traumatised bowel. Representative sections of rectal biopsies from a control subject and Crohn's patient are shown. These are stained for myeloperoxidase (MPO), as a marker of neutrophils, or IL-8. **(A-D)** Untraumatised bowel contained few cells of either type. In the control subject, trauma induced a substantial neutrophil influx **(E)** and increase in IL-8-producing cells **(G)**. This response was attenuated in Crohn's patients **(F,H)**.

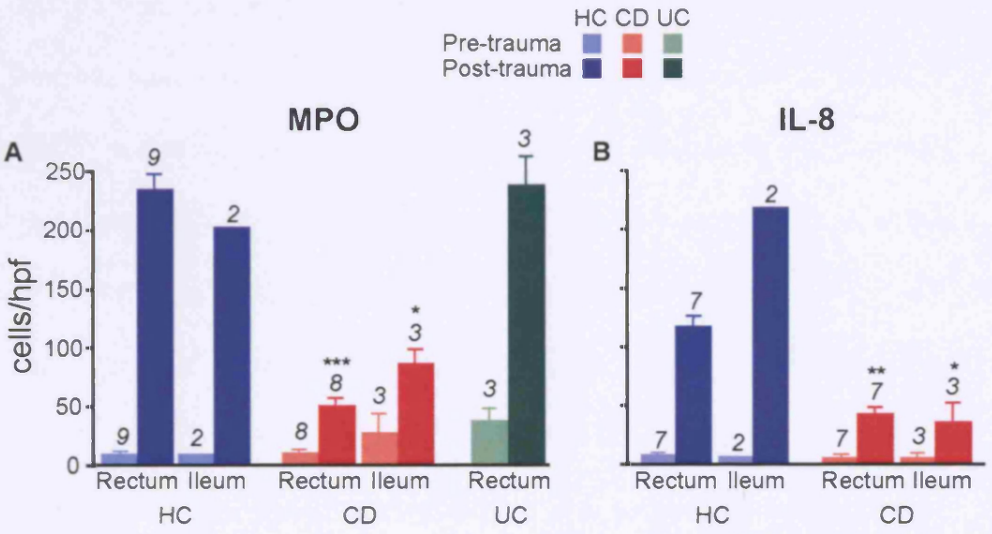


Figure 3.4 Impaired acute inflammation in Crohn's disease. Number of cells staining positive for **(A)** myeloperoxidase (MPO) or **(B)** IL-8 in specimens from different subject groups. In each case, the numbers of cells (mean + SEM) are shown. There was no significant difference in the degree of neutrophil influx between HC and UC patients. Numbers of subjects are shown, significance values compared to post-trauma HC.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients; hpf: high power field.
** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$*

biochemical evidence of disease activity. There was a normal augmentation ($P < 0.001$) in response to trauma (Fig. 3.4A).

3.3 Discussion

3.3.1 Impaired acute inflammation in the bowel in Crohn's disease

A new method was developed to study the acute inflammatory response in the bowel. Whereas analogous techniques in the skin rely on abrasive[387], suction[388] or chemical[389] blister techniques to create new lesions, these were inappropriate for this site. Conversely, biopsy created lesions visible for study at 6 h, in which a vigorous inflammatory response was evident in control subjects.

The degree of neutrophil influx was highly consistent amongst non-inflammatory bowel disease controls. In individuals in whom paired sites in the rectum were biopsied, the inflammatory responses in sections from traumatized bowel were equally intense. Similarly, in individuals in whom both the rectum and ileum was biopsied, the magnitude of the inflammatory response was highly correlated at each site. These observations support the intra-individual reproducibility of this technique.

Using this technique, we found that Crohn's patients exhibited a major impairment in recruiting neutrophils to the bowel. Although this had previously been demonstrated in the skin[232,233], this is the first proof that the same applies to the site predominantly affected in Crohn's disease. All patients except one were in complete remission and off medication (and had been so for at least 2 months prior to study), rendering these highly improbable confounding influences. The failure of migration was observed in all patients regardless of *CARD15* genotype, indicating either that this molecule is not relevant for this process or that wild

type individuals possess mutations elsewhere along the same or a related pathway.

The impairment applies both to the large and small bowel, as well as the skin, suggesting a systemic abnormality in the inflammatory response that is not organ specific. Impaired neutrophil accumulation could result in reduced clearance of any debris penetrating the bowel wall, underlying the granuloma formation frequently observed in this disease. The predilection for lesions to develop in the bowel may relate to the numbers of bacteria present at this site, compared to other organs in the body.

The pattern observed in ulcerative colitis was very different. Increased numbers of neutrophils were present at baseline, despite a macroscopically normal mucosal appearance and absence of indicators of disease activity. Following trauma, these individuals displayed a normal augmentation of mucosal neutrophils, suggesting that the impairment is specific to Crohn's disease and not secondary to any chronic inflammation affecting the bowel.

Trauma to the bowel in non-inflammatory disease controls was also accompanied by substantial induction of IL-8, predominantly by interstitial macrophages. Such pro-inflammatory cytokines are central to the initiation and co-ordination of acute inflammation. IL-8 itself functions as a potent neutrophil chemoattractant[390], and failure of its production may play a primary role in the reduction in their migration into traumatised Crohn's bowel.

3.3.2 Further characterisation of the defect in acute inflammation

Although the serial biopsy technique allows investigation of the primary site affected in Crohn's disease, the bowel remains relatively inaccessible for study.

Elucidating the role of the CARD15 protein in this response would not be feasible, due to the impracticality of administering the proposed ligand[288,289]. Topical application of MDP by instillation in the rectum would not necessarily prove helpful: a thrombus rapidly forms over the biopsy site, the permeability of which would be difficult to measure without using a radiolabeled compound. Similarly, intra-mucosal injection would be difficult to control and might induce unwanted side effects that would limit its application. Given these considerations and the systemic nature of the defect, the skin window model was employed to further characterize the acute inflammatory response (Chapter 4).

Chapter 4: Skin Windows

4.1 Introduction

The results presented in the previous chapter replicated the earlier finding of diminished neutrophil accumulation in Crohn's disease following trauma to an epithelial surface[232]; this time in the gastrointestinal tract (see *3.2.4*) in which there was an association with reduced local IL-8 production. The defect therefore appears to have a systemic component. Consequently, in view of the difficulties of accessibility for study inherent with the intestinal tract, the skin window model was used to pursue these investigations and elucidate the influence of *CARD15*.

Insult or injury to the body elicits an acute inflammatory response. This serves to kill microbes, remove debris and promote healing, and depends on the extravasation of leukocytes and serum proteins. Numerous chemical mediators regulate these events[391,392], produced first by resident immune cells in the tissues then by activated cells that accumulate at the site of insult. Abnormalities in this early response have been linked to the pathogenesis of several diseases[232,393-395], and its modulation by pharmacological agents has important therapeutic implications.

The "skin window" technique permits investigation of the acute inflammatory response *in vivo* in humans. This typically involves the creation of a lesion in the stratum corneum of the skin using a surgical scalpel[387], a high-speed drill[396] or tape stripping[397]. Whilst these allow examination of leukocyte emigration, they remain unsuitable for measuring the concentrations of secreted mediators. In addition, exogenous chemicals cannot be applied reliably. Others

have used negative pressure suction to raise blisters[388], although these contain only a small volume of fluid with few cells. Formation of large volume blisters can be provoked by topical application of cantharidin (derived from the *Meloidae coleoptera* beetle)[389], aspiration of which enables the collection of high numbers of cells and quantification of inflammatory mediators. Cantharidin, however, acts by inhibiting protein phosphatase-1 and protein phosphatase-2α[398], confounding its use in studying cell function and signalling.

To overcome these difficulties, the skin window technique was refined in this study. Abrasions were overlaid with filter papers onto which cells migrated and soluble mediators were absorbed. Impregnation of these dressings with inflammatory modulators enabled assessment of their effects on the acute response.

4.2 Results

4.2.1 Acceptability

Skin windows were created in 6 healthy controls, 13 Crohn's patients, 3 patients with ulcerative colitis and 3 patients with rheumatoid arthritis (Appendix 1: Tables A1.4-A1.7 respectively). All subjects were in clinical remission. Crohn's patients were taking no immunosuppressive medication and had not done so for at least the past two months, and none showed any evidence of malnutrition.

The technique did not interfere with the daily activities of participants. Dermal abrasion (Fig. 4.1A) was used to create windows of a standard area (Fig. 4.1B). This process was described as uncomfortable but not painful until the capillary beds were visualized, at which point abrasion was discontinued. The windows were not painful over the course of the experiment, although a minority

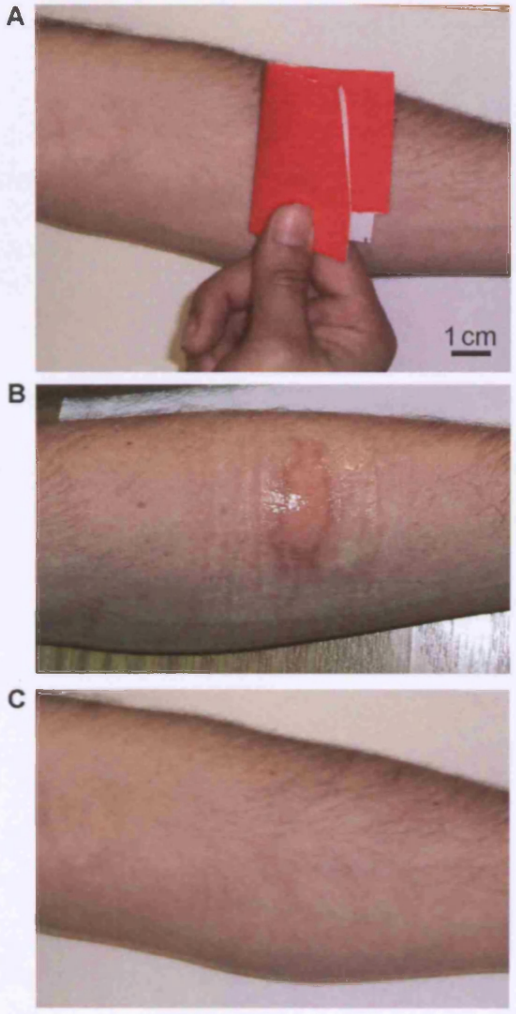


Figure 4.1 Skin window technique. **(A)** Skin windows were created by dermal abrasion. **(B)** This was used to create lesions with a standard area of 3 cm^2, which were then overlaid with filter papers. **(C)** Windows healed within 14 days.

of subjects described minor discomfort overnight when pressure was inadvertently applied during sleep. No bleeding occurred into the windows, and re-epithelialization occurred within 14 days (Fig. 4.1C). A minority of healthy subjects reported hypopigmentation at the abrasion sites, lasting no more that 2 months. Interestingly, this appeared to persist slightly longer in Crohn's patients, which may reflect impaired wound healing. There were no additional side effects. In terms of acceptability, therefore, this method is comparable to the alternative established techniques.

4.2.2 Neutrophil influx is diminished in Crohn's disease

In healthy controls, the majority of cells infiltrating skin windows by 6 h and 24 h were neutrophils (Fig. 4.2A). These still predominated at 48 h, although an increased proportion of monocyte/macrophages and lymphocytes had accumulated. In concordance with previous studies[232-234], we observed a relative reduction in neutrophil emigration in Crohn's patients at 6 h and 24 h (Fig. 4.2A). This appeared to represent a delay in migration, as neutrophil influx in these individuals was proportionally greater at 48 h. Direct measurement of myeloperoxidase concentrations in skin window cell lysates from 24 h windows (Fig. 4.2B) confirmed that the relative reduction in neutrophils was due to an absolute deficiency ($P = 0.2 \times 10^{-6}$) rather than an increase in the numbers of other leukocytes, which migrated normally.

The grossly diminished neutrophil efflux into skin windows (previously reported in Crohn's patients after 5 h) was therefore also present after 24 h with an approximately 50% reduction in patients. This might be an underestimate: the

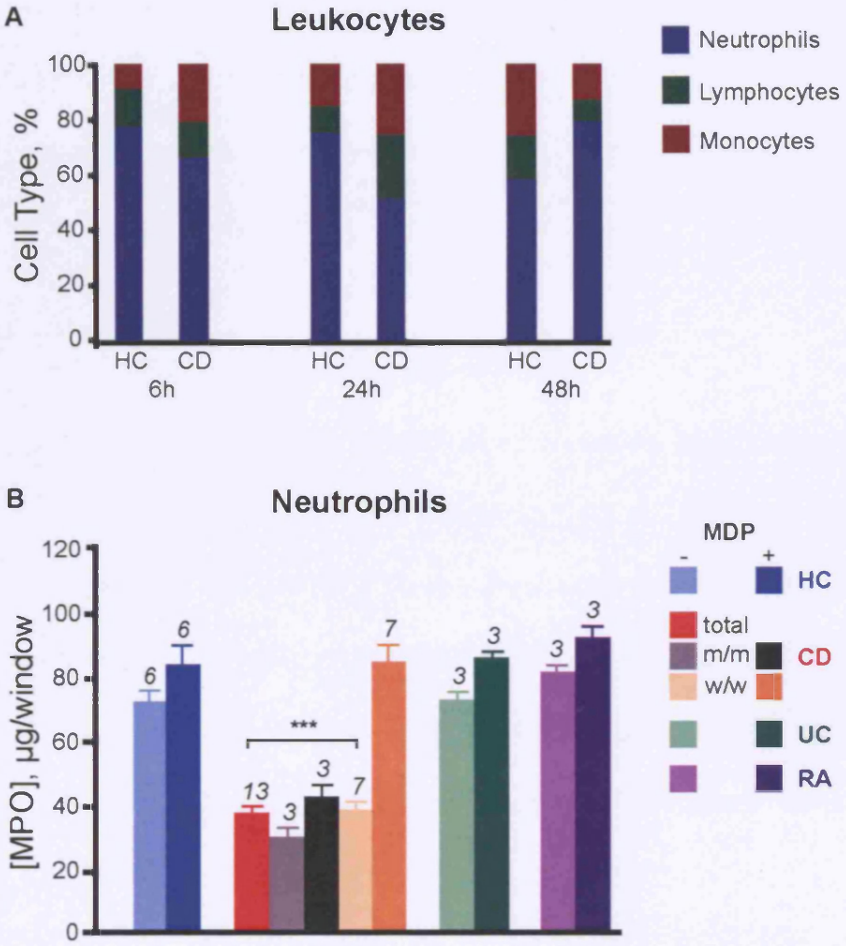


Figure 4.2 Leukocytes emigrating to the site of skin trauma. **(A)** Differential leukocyte composition in 3 healthy controls and 3 Crohn's patients over a 48 h time course. **(B)** Neutrophil emigration into skin windows was impaired in Crohn's disease, irrespective of *CARD15* genotype. Topical application of MDP corrected neutrophil recruitment only in *CARD15* wild type (*w/w*) patients, and not in homozygotes/compound heterozygotes (*m/m*). In each case, the numbers of cells (mean + SEM), the numbers of subjects in each group and the significance levels compared to healthy controls are shown.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients; RA: rheumatoid arthritis patients; MPO: myeloperoxidase

**** $P < 0.001$*

whole filter paper surface area was covered with cells in control subjects by this time, suggesting that the assay might be saturated.

A similar degree of impairment was observed in patients who were homozygous/compound heterozygous for *CARD15* polymorphisms (*m/m*) as in wild type patients (*w/w*). Neutrophil emigration was normal in subjects with ulcerative colitis and in those with another chronic inflammatory disease not affecting the bowel, rheumatoid arthritis.

4.2.3 Normal production of early inflammatory mediators

The concentrations of a panel of early inflammatory mediators were assayed in fluid taken from skin windows 30 min following trauma. These included histamine, prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4), activated complement component C3a (measured as C3a-desArg, the stable product into which C3a is rapidly converted that allows reliable quantification of C3 activation[399]), and IL-1β. All were similar in healthy controls and Crohn's patients (Fig. 4.3A).

4.2.4 Diminished chemokine production in Crohn's disease

To identify cytokines present in skin window exudates, they were assayed on antibody arrays. Of the 42 cytokines examined (see Table 2.2), only IL-8 and IL-1β were detected (Fig. 4.3B). Exudates from Crohn's patients had levels of these cytokines approximately 50% ($P < 0.001$) of those in control subjects (Fig. 4.3C,D). TNF-α and TGF-β were also assayed, as control cytokines. TNF-α was detectable in only 50% of skin windows (Fig. 4.3E), with no difference between

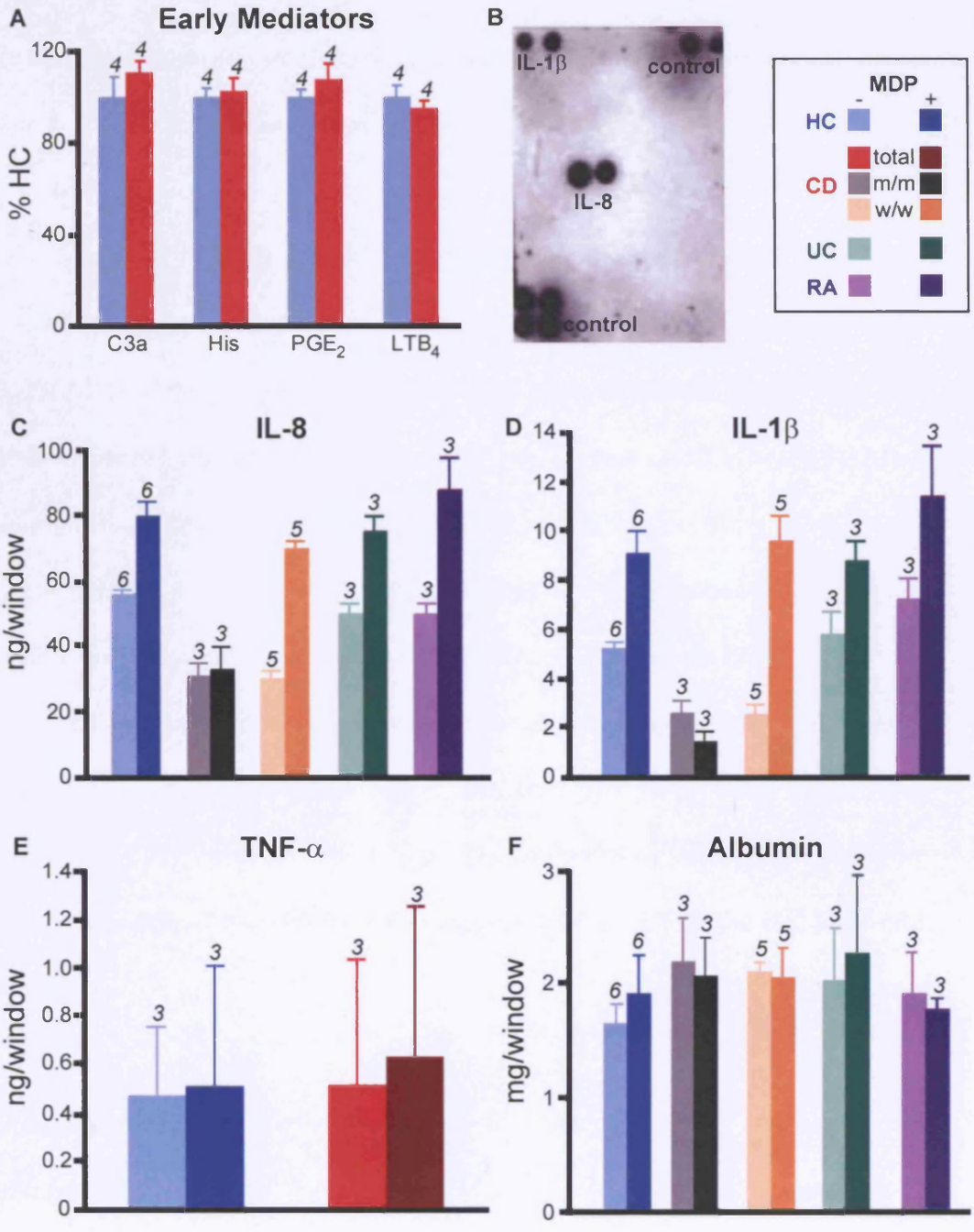


Figure 4.3 Production of inflammatory mediators in skin windows. **(A)** A panel of early inflammatory signals was normal 30 min following trauma in CD. **(B)** A representative membrane arrayed with antibodies against 42 different cytokines (each in duplicate), probed with fluid from a HC skin window 24 h after trauma, reveals predominant production of IL-8 and IL-1β. Secretion of **(C)** IL-8 and **(D)** IL-1β was diminished in CD at 24 h, irrespective of *CARD15* genotype. Topical application of MDP augmented cytokine production in *CARD15* wild type (*w/w*) subjects but not in homozygous/compound heterozygous (*m/m*) patients. **(E)** TNF-α secretion was similar and minimal in all subjects, and not affected by MDP. **(F)** Exudation of albumin was equivalent in each window created, indicating that differences observed do not reflect differential traumatisation. Means + SEM and numbers of subjects in each group are shown.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients; RA: rheumatoid arthritis patients; MPO: myeloperoxidase

healthy controls and Crohn's patients. TGF-β activity could not be detected in any sample. Samples from all subject groups were prepared and processed on the same day, such that differences are unlikely to reflect intra-assay variability.

4.2.5 Exogenous IL-8 corrects the defect in neutrophil accumulation

To determine whether depressed neutrophil emigration in Crohn's patients might be due to low levels of the potent neutrophil chemoattractant IL-8[390], two skin windows were performed on 3 patients (2 *w/w*, 1 *m/m*) with recombinant IL-8 applied to one abrasion. In each patient, this resulted in doubling of neutrophil emigration ($P < 0.05$; Fig 4.4A) and augmented IL-1β production ($P < 0.05$; Fig. 4.4C), to levels observed in healthy subjects exposed to trauma alone.

To confirm the effects of exogenous IL-8 on neutrophil emigration in healthy individuals, a separate experiment was performed using larger filter papers (35 x 15 mm) to circumvent the confounding effects of saturation. Myeloperoxidase ($P < 0.05$; Fig.4.4B) and IL-1β ($P < 0.05$; Fig. 4.4D) were both increased.

4.2.6 MDP corrects the defect in CARD15 wild type patients

Application of MDP to skin windows amplified IL-8 and IL-1β secretion in healthy, ulcerative colitis and rheumatoid arthritis subjects, and also in *w/w* Crohn's patients (Fig. 4.3C,D) in whom neutrophil migration was concomitantly normalised (Fig. 4.2A). In contrast, neither cytokine secretion nor neutrophil emigration was augmented in *m/m* patients (Fig. 4.2A; Fig. 4.3C,D).

For comparability with previous parts of the study, regular sized filter papers were used to examine neutrophil emigration in control subjects (Fig.

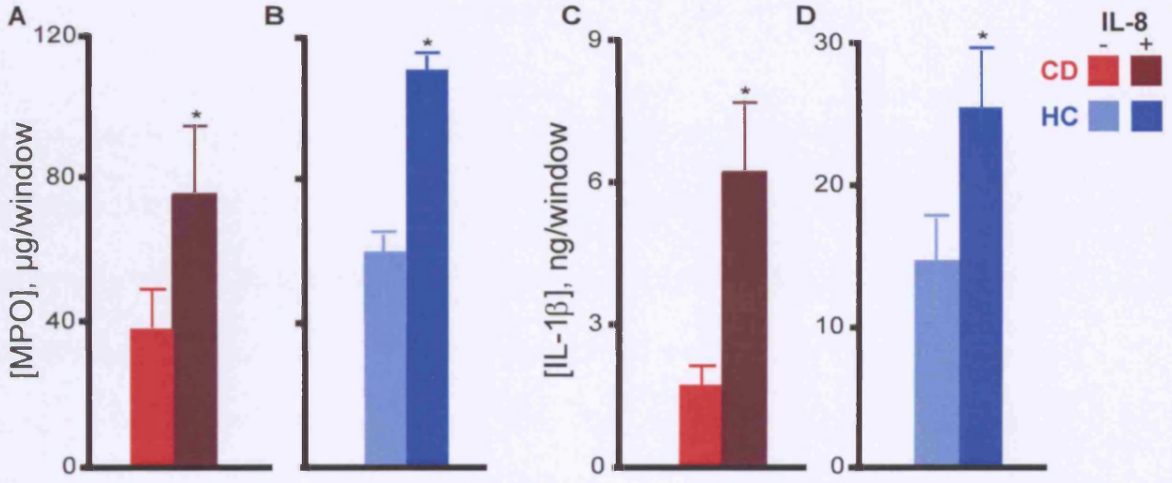


Figure 4.4 Augmentation of neutrophil emigration and IL-1β secretion into skin windows by topical IL-8. Myeloperoxidase content was increased in CD patients **(A)** and HC subjects **(B)**. IL-1β levels also rose in these groups **(C,D)**. Mean + SEM shown.

HC: healthy controls; CD: Crohn's patients; MPO: myeloperoxidase

** P < 0.05*

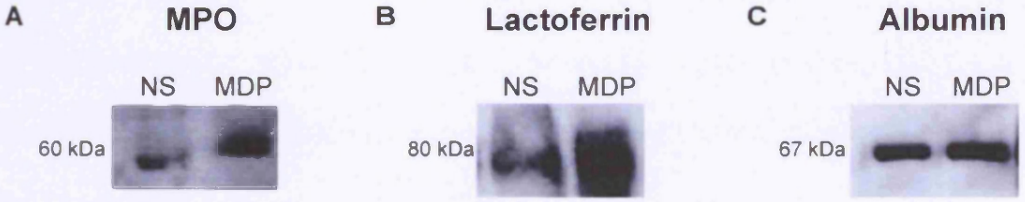


Figure 4.5 Confirmation of increased neutrophil emigration induced by topical MDP into skin windows of healthy controls. Representative immunoblots (equal protein loading) demonstrate increased content of the neutrophil markers MPO **(A)** and lactoferrin **(B)**, but equal albumin exudation **(C)**, in the presence of MDP compared to trauma with normal saline (NS) alone.

NS: normal saline; MDP: muramyl dipeptide; MPO: myeloperoxidase

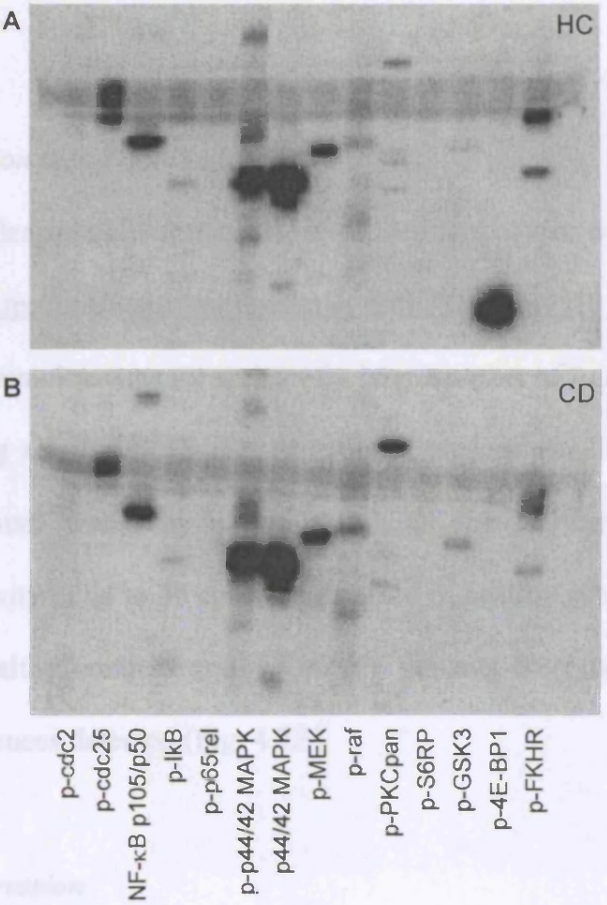
4.5A-C). In these skin windows, MDP led to an increase in the amounts of myeloperoxidase ($P < 0.05$ by biochemical measurement for all controls) and lactoferrin[400] but not albumin. This increase was more modest than that seen with IL-8, but could have been confounded by saturation.

4.2.7 Observed differences do not reflect differential traumatisation

Albumin was measured in each sample as an indicator of the extent of trauma induced to create skin windows. This was equivalent in the different subject groups and following topical application of immunomodulatory agents. These albumin concentrations, combined with the equivalent levels of early inflammatory mediators (Fig. 4.3A), suggests that differences in cell recruitment and cytokine production cannot be explained by differential degrees of skin abrasion.

4.2.8 Abnormal phosphorylation of cytoplasmic signalling pathways

The phosphorylation status of proteins involved in major signalling pathways was examined in cytoplasmic extracts of leukocytes from the exudates. In cells from 5 healthy individuals, with the panel of antibodies applied, phosphorylation of FKHR was observed at 6 h; 4E-BP1, p38 and p44/p42 MAP kinases, AKT, protein kinase C (PKC) and SAPK/JNK at 24 h; and AKT and PKC at 48 h (Fig. 4.6A). In 5 Crohn's patients (Fig. 4.6B), phosphorylation levels of the majority of these proteins were equivalent. A few differences were apparent, most strikingly hyperphosphorylation of PKC, specifically PKCα/β (Fig. 4.6C). This was observed in all patients at each time point. There were no differences in levels of phosphorylation of PKCδ, PKCζ/λ PKCθ or PKCμ. Two patients



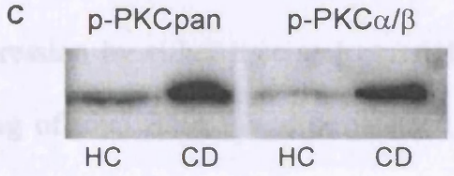


Figure 4.6 Altered phosphorylation of proteins in major cytoplasmic signalling pathways in Crohn's disease skin windows. Representative immunoblots (equal protein loading) illustrate some of the differences observed between healthy **(A)** and Crohn's **(B)** subjects. The most consistent alteration was hyperphosphorylation of PKC, observed in all Crohn's patients at each time point. This was specific to PKCα/β **(C)**.
HC: healthy control; CD: Crohn's patients

showed reduced phosphorylation of 4E-BP1, whilst that of p70S6 kinase was increased. A third patient had decreased phosphorylation of Akt and a fourth of p38 MAP kinase.

4.2.9 Transcription factor activation

DNA-bound nuclear protein extracts from exuded leukocytes were hybridized to arrays (see http://panomics.multipath.net/prdf/PD_Array_1_with_ap.pdf for layout) to allow simultaneous screening of a large number of transcription factors for DNA-binding activity. Sufficient amounts of nuclear proteins were obtained to allow maximum sensitivity of the assay. Of the 54 transcription factors assayed, the activity of up to 44 could be detected in healthy subjects (Fig. 4.7A). A total of 4 healthy controls and 4 Crohn's patients were compared, but no consistent differences detected (Fig. 4.7B).

4.2.10 Gene expression

Sufficient quantities of mRNA could also be extracted to permit extensive analysis of gene expression by either reverse transcriptase-PCR or microarray. Approximately 1-2 μg of total RNA could be isolated from one-eighth of the total amount of exuded cells, which would be sufficient to analyze the expression of over 50 genes. To demonstrate the viability of such an assay, expression of the monocyte cell marker CD14 and GAPDH were determined in 24 h extracts from 2 healthy controls and 2 Crohn's patients (Fig. 4.7C). Similar expression of both indicated equivalent levels of monocyte influx.

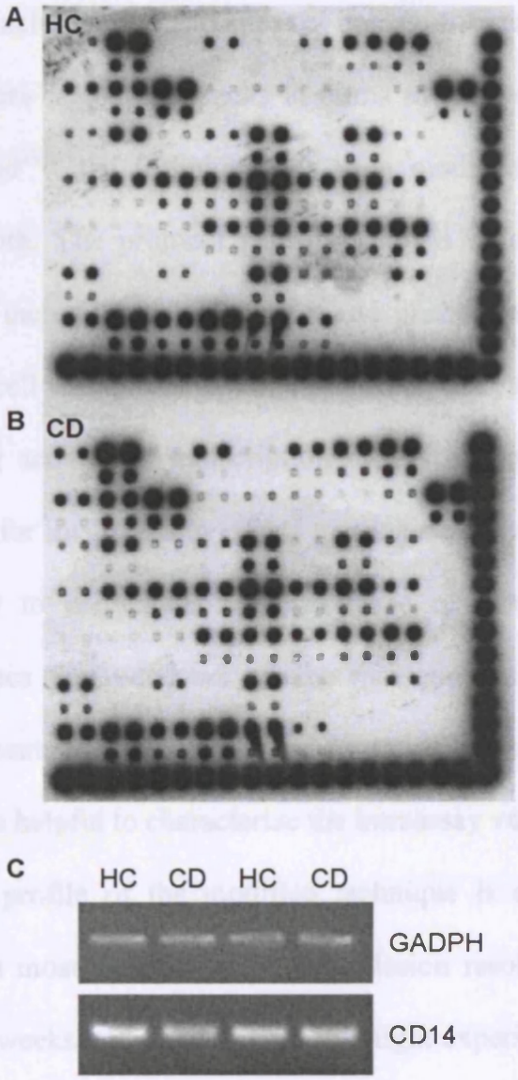


Figure 4.7 Investigation of transcription factor activation and gene expression in skin windows. Representative DNA-bound protein arrays from **(A)** healthy and **(B)** Crohn's subjects. No consistent differences were observed. **(C)** RT-PCR demonstrates the viability of gene expression analysis in leukocyte extracts from skin windows using the technique developed in this study. Equivalence of CD14 levels in HC and CD subjects confirms equal extravasation of monocytes between the two groups at 24 h.

HC: healthy controls; CD: Crohn's patients

4.3 Discussion

4.3.1 Modifications to the skin window technique

The skin window technique has previously been used to investigate the acute inflammatory response, including in the context of human disease. All previously described methods possess major drawbacks in terms of recovery of cells and inflammatory tissue fluid[389]: the technique has been modified here to allow reliable collection of both. The protocol permits analysis of all facets of the inflammatory response, including cellular influx and phenotype, production of secreted mediators, and cell function in terms of activation of cytosolic signalling pathways, DNA-binding activity of transcription factors and gene expression. The system is also ideal for investigating effects of exogenous mediators, as they can be applied directly to the abrasion. Equivalence of albumin and early mediator release indicates that windows can be fashioned in a reproducible manner. Further experiments in which two saline treated windows are performed on each subject would be helpful to characterize the intraassay variability[401].

The side effect profile of the modified technique is similar to those previously described. In most control subjects, the lesion resolved completely within approximately 3 weeks, although a minority might experience continuing hyperpigmentation for up to 1 year. An interesting observation was that in Crohn's patients, healing tended to require slightly more time with an increased frequency of abnormal pigmentation. Although not objectively measured in this study, it would be consistent with the impaired wound healing described in this disease[259].

4.3.2 Characterisation of skin window inflammatory contents

Cells migrating into the skin windows in healthy subjects were predominantly neutrophil leukocytes, classically the first line of cellular defence against infection[205]. These were partially replaced by mononuclear phagocytes over the subsequent 48 h, which contribute to the removal of neutrophils and healing after initial clearance of debris[258]. The differential separation of these cells and their further characterisation using the technique developed here has been described elsewhere[402].

Cellular extravasation was accompanied by the production of high concentrations of pro-inflammatory mediators, at similar levels amongst control subjects. Early inflammatory mediators were elevated 30 min after trauma, at equivalent levels in all subjects. At 24 h, the predominant cytokines were IL-8 and IL-1β, as detected using cytokine protein arrays. TNF-α was also demonstrable at low levels in some windows using an ELISA, by virtue of its greater sensitivity than the membrane array. It is acknowledged that some cytokines, such as IFN-γ, may be difficult to elute from filter papers[401].

4.3.3 Reduced chemokine production in Crohn's disease underlies diminished neutrophil migration

Impaired neutrophil migration into skin windows in Crohn's disease was previously reported at 6 h following skin trauma[232]. This finding was replicated here, in correspondence with the defect observed in the bowel (see *3.2.4*). The study was designed to minimize potential confounding factors, such as use of immunosuppressive medications. In active inflammation, reduced neutrophil migration could theoretically arise from their sequestration in gastrointestinal

lesions. Patients in this study, however, had inactive disease (by clinical and biochemical criteria), and the impairment was not observed in other chronic inflammatory disorders, suggesting that these are unlikely explanations.

Although an *in vivo* impairment in neutrophil chemotaxis has previously been described, the mechanism remained speculative. Normal migration *in vitro* to casein, zymosan-activated serum or bacterial chemotactic factors[106] suggested an extrinsic defect. An inhibitory chemotactic factor was demonstrated in the serum of Crohn's patients in *ex vivo* experiments[235], although this inhibition was also present to a lesser degree when using normal serum and found equally in Crohn's disease and ulcerative colitis, varying with disease activity. Although a definitive identification of the factor was not made, the biochemical properties implicate immunoglobulins. It was postulated that increased circulating levels of these proteins in active Crohn's disease could underlie the failure in skin window chemotaxis.

An alternative mechanism is proposed here based on the observation of diminished concentrations of chemotactic cytokines that parallel the impairment in neutrophil recruitment. The finding of reduced levels of IL-8, an extremely potent neutrophil attractant[390], could readily explain the observed defect. The impairment was seen in patients with inactive disease and not in the inflammatory disease controls, which also argues against the underlying problem relating to inhibition by circulating immunoglobulins.

In the few patients treated here, exogenous IL-8 corrected the defect in neutrophil migration indicating that the chemokine receptors are intact. This suggests that the impairment does relate to lack of the appropriate stimulus in these subjects, although other patients could conceivably possess abnormal IL-8

receptors or downstream signalling pathways. That neutrophil recruitment can be corrected highlights a potential therapeutic approach, should this prove the underlying pathogenic mechanism.

4.3.4 CARD15 polymorphisms exacerbate diminished neutrophil migration

The normal function of CARD15 remains poorly described. It is expressed within leukocytes, including monocytes, macrophages[279] and granulocytes[281]. Most studies have focused on its role in gene expression and cytokine production[288,403], whereas none have examined its influence on chemotaxis *in vitro* or *in vivo*.

Crohn's patients in this study were subdivided into those homozygous/compound heterozygous for *CARD15* polymorphisms and those without any of the three common variants. The two groups could not be distinguished on the basis of their response to epithelial trauma alone in this model. This indicates either that CARD15 does not contribute to neutrophil migration and the establishment of acute inflammation to a stimulus of this nature, or that other mutations elsewhere in this pathway or in other pathways ultimately produce the same phenotype in wild type patients.

A difference between subjects was observed following topical administration MDP (the CARD15 ligand), never previously used *in vivo* in humans in this manner. In wild type patients, this increased concentrations of IL-8 and IL-1β in skin windows, leading to correction of neutrophil numbers to normal levels. In patients with the polymorphisms there was no response. This result is very pertinent for resolving the conflicting theories concerning the role of CARD15. Many attempts have been made to reconcile the originally reported

apparent loss-of-function with the chronic inflammation characteristic of active Crohn's disease. The leading suggestions remain that the polymorphisms either lead to impaired induction of anti-inflammatory cytokines[292]; that lack of negative regulation by CARD15 engenders unrestrained activation of the adaptive immune system[294]; or that there is a toxic gain-of-function[293]. This study demonstrates that the opposite appears to be the case: CARD15 is normally pro-inflammatory *in vivo*, an action abrogated by the polymorphisms.

This is liable to pertain to the situation in the gastrointestinal tract. Following a mucosal breach, the luminal contents (including bacteria and their products) will ingress into the bowel wall. In the general population, there will be a normal distribution in the magnitude and quality of the inflammatory response that ensues. Some individuals will be towards the low end of this spectrum, and might be at risk of developing Crohn's disease. Having responded poorly to the mucosal breach, however, there will then be additional stimulation by the influx of luminal contents, such as MDP acting through CARD15. The subsequent degree of augmentation of cytokine production will determine whether the initial defect is adequately compensated and clearance of debris sufficient. If this is not the case, then suboptimal phagocyte recruitment could result in persistence of exogenous material, granuloma formation and chronic inflammation.

It is tempting to speculate that this mechanism explains the predisposition of *CARD15* polymorphisms to Crohn's disease affecting the small bowel. MDP is a small 493 Da molecule, found at high concentrations in the terminal ileum[404]. A highly diffusible molecule such as this would be expected to exert greatest influence in the region of the bowel containing fluid luminal contents with heavy bacterial colonisation. Another prediction of this model would be that

polymorphisms in other pathogen recognition receptors also predispose to Crohn's disease, perhaps specific to other intestinal regions. This appears to be borne out by preliminary observations hinting at susceptibility variants in the *CD14*[323] and *TLR4*[324] genes.

4.3.5 Signalling pathways in Crohn's disease

The phosphorylation status of a number of major signalling proteins was also analyzed, the profiles of which appeared highly consistent amongst control subjects. A central molecule indicated from this study was Akt, which has been documented to activate many of the other signalling molecules found to be phosphorylated here[405,406]. Akt plays a critical role in cell survival by inhibiting apoptosis through phosphorylation of intermediaries such as FKHR[407] and MEK/ERK[408]. It can also activate other proteins including 4E-BP1, which promotes translation of mRNA[409].

A preliminary search for any signalling abnormalities in exuded leukocytes in Crohn's patients revealed that phosphorylation levels of most proteins were unchanged. Of the few differences apparent, the most striking and consistent was hyperphosphorylation of PKCα/β. This was observed at all time points, including at 48 h when neutrophils were proportionally more prevalent in patients than in the controls. Consequently, a genuine abnormality in activation of this pathway during acute inflammation is implied, as opposed to altered levels simply reflecting differential leukocyte migration.

Other variations in some patients included decreased levels of phosphorylation of 4E-BP1, Akt and p38 MAP kinase, and increased phosphorylation of p70S6 kinase. The heterogeneity may relate to differences in

the underlying molecular aetiology that probably exists between patients. Changes in phosphorylation were more pronounced than the deficits in neutrophil exudation. This, and the similarity in levels of other proteins and their phosphorylation ratios between controls and Crohn's patients, argues against differences relating solely to altered cellular distributions. To finalize this issue, exuded leukocytes could be fractionated in future using standard methodology[402].

The functional significance of the changes observed in Crohn's disease remains unclear and merits further investigation. Altered phosphorylation of molecules involved in protein translation, including p70S6 kinase[410]and 4E-BP1[409], may contribute to defective production of inflammatory mediators. Other mechanisms may be more complex: PKC interacts with a broad range of cell signalling pathways with pleiotropic effects[411]. This could be studied by examining the function of exuded leukocytes from controls and patients *ex vivo*, in the presence of pharmacological agonists and inhibitors[412] of pathways of interest.

4.3.6 The cellular basis for defective cytokine production

The data presented in this chapter confirm the systemic nature of the defect in neutrophil recruitment and implicate a failure of cytokine production as an important causal mechanism. In the rectal biopsy model (see *3.2.4*), considerable IL-8 was produced by healthy interstitial mononuclear phagocytes; *CARD15* is also expressed predominantly in these cells under normal conditions[280]. Consequently, macrophage responses were studied *in vitro* to determine their ability to produce mediators involved in the initiation of the acute inflammatory response in Crohn's disease (Chapter 5).

Chapter 5: Macrophage Cytokine Production

5.1 Introduction

Macrophages are mononuclear phagocytes that contribute substantially to innate immune defences[413]. They possess a high capacity for uptake of micro-organisms and particulates[414], similar to neutrophils[205]. In contrast, some are resident in tissues in a physiological role[415] and their circulating precursors are recruited during a later phase of the inflammatory response[396]. They exert a more limited microbicidal action[204,416], often requiring co-stimulation by lymphocytes[417], and subserve broader functions including antigen presentation[418]. Through the expression of numerous pathogen recognition receptors, including the TLRs[300,419], they play a critical role in detection of potential pathogens within the tissues. They then co-ordinate the ensuing inflammatory response, mediated by secreted cytokines and direct interactions with other leukocytes[420].

CARD15 is principally expressed in mononuclear phagocytes[279], where it is thought to act as a pathogen recognition receptor. It was originally believed to respond to bacterial LPS, on the basis of experiments in transfected cell lines. It was subsequently demonstrated that the cells were responding not to LPS but to contaminating peptidoglycan, another component of the bacterial cell wall from which the LPS had been prepared[288,289]. Digestion of purified peptidoglycan into smaller muropeptides demonstrated that MDP constituted the minimum unit recognized by CARD15.

The normal cellular function of CARD15 remains poorly understood. In humans *in vivo* it appears to exert a pro-inflammatory influence (see Chapter 4), augmenting production of IL-8 and IL-1β, and consequently neutrophil

recruitment (see *4.2.6*). Some further aspects of function can be deduced by examining patterns of gene expression in MDP-stimulated macrophages from individuals with and without *CARD15* polymorphisms. Inferences may also be drawn from computer models of its predicted protein structure.

Conversely, the abnormalities in neutrophil recruitment and cytokine production observed in previous chapters (see *3.2.4*, *4.2.2* and *4.2.4*) appeared unrelated to *CARD15* genotype. Correction of leukocyte numbers in Crohn's skin windows by exogenous or increased endogenous IL-8 (see *4.2.5* and *4.2.6* respectively) suggests that diminished production of this cytokine might play a central role. IL-8 predominantly derives from macrophages[421], although infiltrating neutrophils can provide a further source[422]; similarly in the intestinal serial biopsy model the majority of IL-8-positive cells were mononuclear phagocytes. Damage to an epithelial surface will provoke release of a large number of pro-inflammatory mediators; consequently, the most physiological agonist to mimic conditions in the skin window would be fluid taken from a site of acute tissue injury.

5.2 Results

5.2.1 CARD15 does not influence LPS-induced IL-12 production

Peripheral blood mononuclear cells were cultured for 5 days, with purification and differentiation of macrophages by adherence (Fig. 5.1A). Cells were more than 95% pure by this time, determined by CD68 staining, with no differences between Crohn's patients and controls. In view of the original reports identifying CARD15 as a LPS receptor, macrophage responses to this agonist were determined initially. LPS is known to induce substantial IL-12 production by

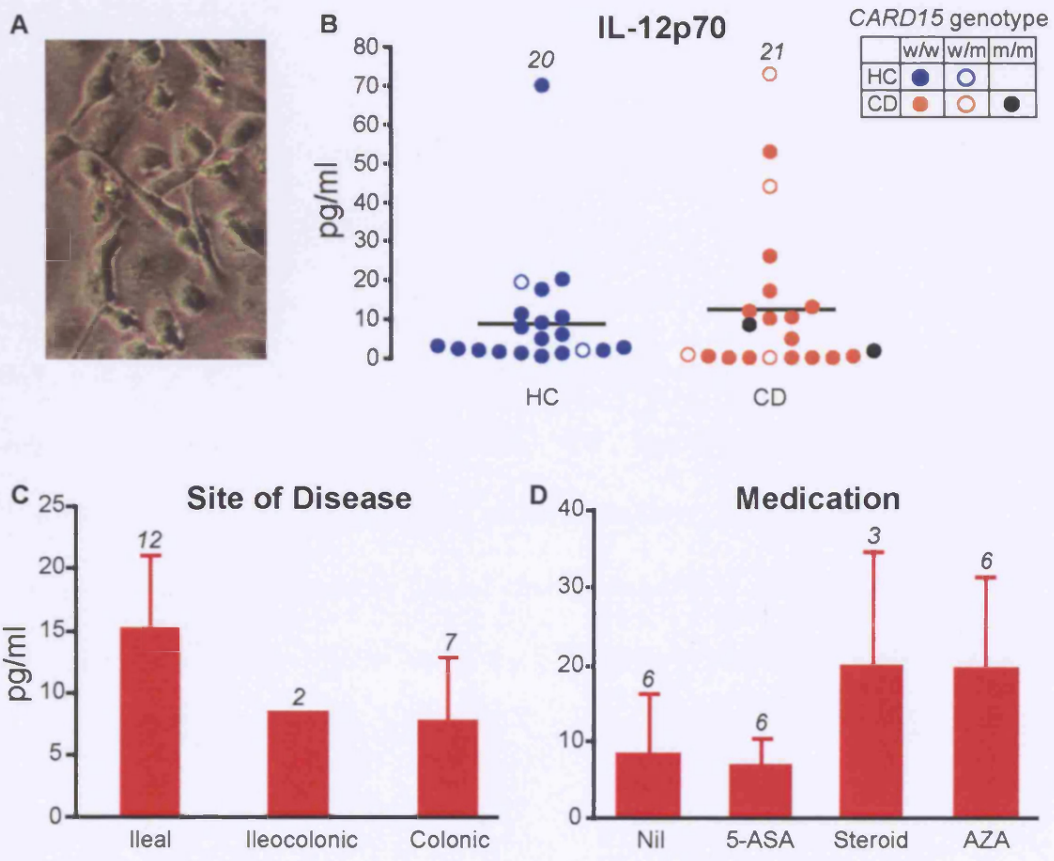


Figure 5.1 LPS-induced IL-12p70 secretion by cultured macrophages. **(A)** Monocytes were cultured for 5 days to allow purification and differentiation into macrophages. IL-12p70 secretion was equivalent in HC and CD, and not related to *CARD15* genotype **(B)**, site of disease **(C)**, or use of medication **(D)**. Mean + SEM shown.

HC: healthy control; CD: Crohn's patients; AZA: azathioprine

w/w: wild type; w/m: simple heterozygote; m/m: compound heterozygote/homozygote

these cells[423], and was therefore assayed in 20 healthy controls and 21 Crohn's patients.

Mean levels of IL-12p70 production were 8.80 pg/ml (standard deviation = 15.50 pg/ml) in control subjects and 12.65 pg/ml (standard deviation = 19.67 pg/ml) in Crohn's patients (Fig. 5.1B). No IL-12 was produced in the absence of stimulation. There was no apparent relationship with *CARD15* genotype (Fig. 5.1B), disease localisation (Fig. 5.1C), any other recorded characteristic (see *2.1.3*), or use of medication (Fig. 5.1D).

An interesting observation was that LPS-stimulated macrophages from 5 Crohn's patients failed to produce detectable levels of IL-12. This was not due to differences in numbers of cells plated, and significant secretion was demonstrated in other samples processed at the same time. This impairment did not segregate with any documented patient characteristic. It is possible that these individuals possess genetic lesions along the LPS-sensing pathway[424], shown to increase susceptibility to Crohn's disease[323,324].

5.2.2 Effects of MDP on macrophage gene expression

Global gene expression profiles induced by MDP were determined in macrophages from 6 *CARD15 w/w* healthy controls. MDP was pure, as determined by mass spectrometry (Fig. 5.2A). Total cRNA was used to interrogate Affymetrix Human Genome U133A microarrays, which cover sequences for 22,000 known genes and expressed sequence tags (ESTs). The duration of stimulation and concentration of MDP used were based on conditions employed in the literature, demonstrated to elicit differential NF-κB activation in *w/w* versus *m/m* cells[288]. The other advantage of these conditions was that they

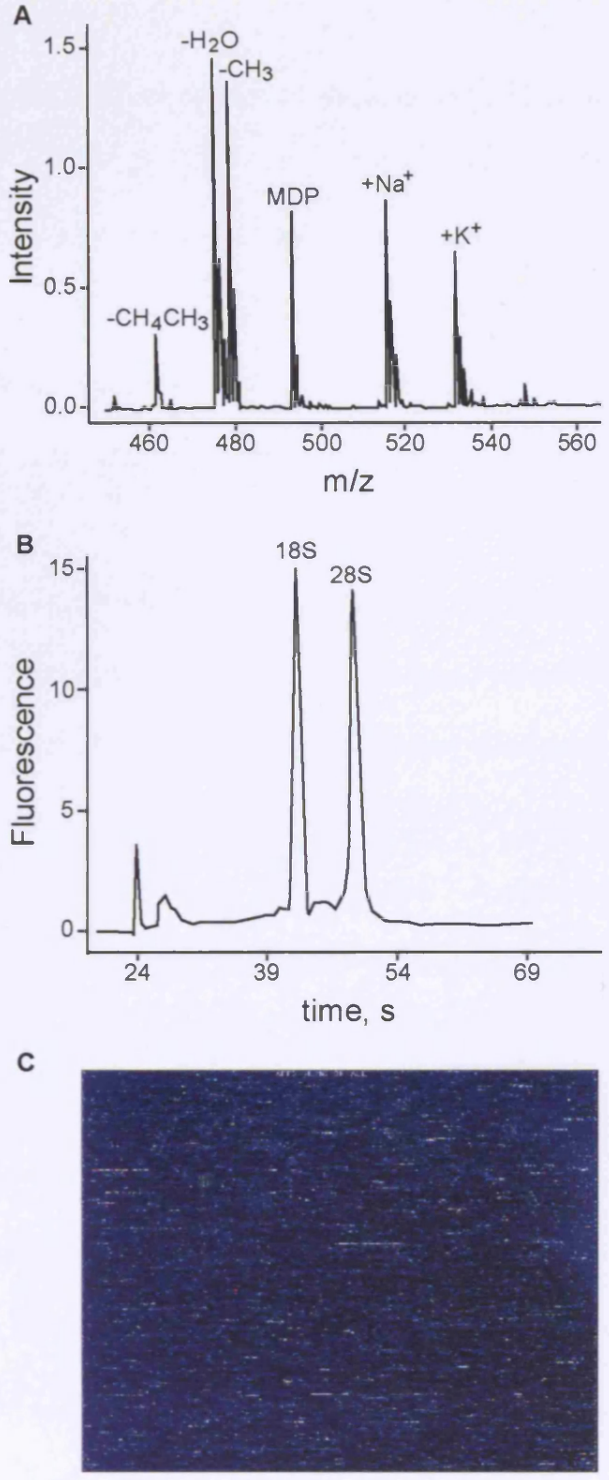


Figure 5.2 Quality control for microarray experiments. **(A)** Purity of MDP was confirmed by mass spectrometry. Different salts of MDP were detected, but no other bacterial cell wall contaminants were present. **(B)** The quality of total RNA was verified using an Agilent Bioanalyzer. Two prominent ribosomal peaks can be seen on a background of level baseline fluorescence, identicating minimal sample degradation. **(C)** Representative microarray following sample hybridization and the detection reaction. High intensity spots indicate positive signals, which were quantified and annotated using an automated reader.

MDP: muramyl dipeptide

corresponded closely to those employed in previous *in vivo* experiments in this study (see *4.2.6*).

The quantity and quality of total RNA isolated from macrophages was confirmed using an Agilent Bioanalyzer (Fig. 5.2B). Following conversion to cRNA, every sample was hybridized to an individual microarray; signals from each (Fig. 5.2C) demonstrated control parameters within recommended limits (see *2.5.4*).

Expression levels of 229 probes were altered by 2-fold or greater in macrophages incubated with MDP compared to the unstimulated state. Of these, approximately two-thirds were induced, and the remainder repressed (Fig. 5.3A,E; Table 5.1 and Appendix 2). Genes up-regulated predominantly encoded pro-inflammatory molecules; these were principally cytokines, amongst which IL-8[390], IL-1β[425] and RANTES[426] featured prominently. Other induced genes encoded cytokine-associated molecules (including indoleamine-2,3-dioxygenase, an enzyme known to potentiate IL-8 production and function[427]); GTP cyclohydrolase-1 (involved in generation of the NOS co-factor tetrahydrobiopterin[428]); molecules contributing to matrix repair (TNFα-induced protein-6 (TSG-6)[429], α1-antitrypsin[430], various metallothioneins[431], and growth factors HB-EGF[432] and VEGF[433]); and molecules that participate in macrophage activation and the respiratory burst (including NADPH oxidase p47phox[434], superoxide dismutase-2[435], C-type lectin[436], CD38[437], interferon-regulatory factor-1[438] and the Fcγ receptor I B1[439]). Important transcripts that were down-regulated included various tissue inhibitors of metalloproteinases (TIMPs)[440].

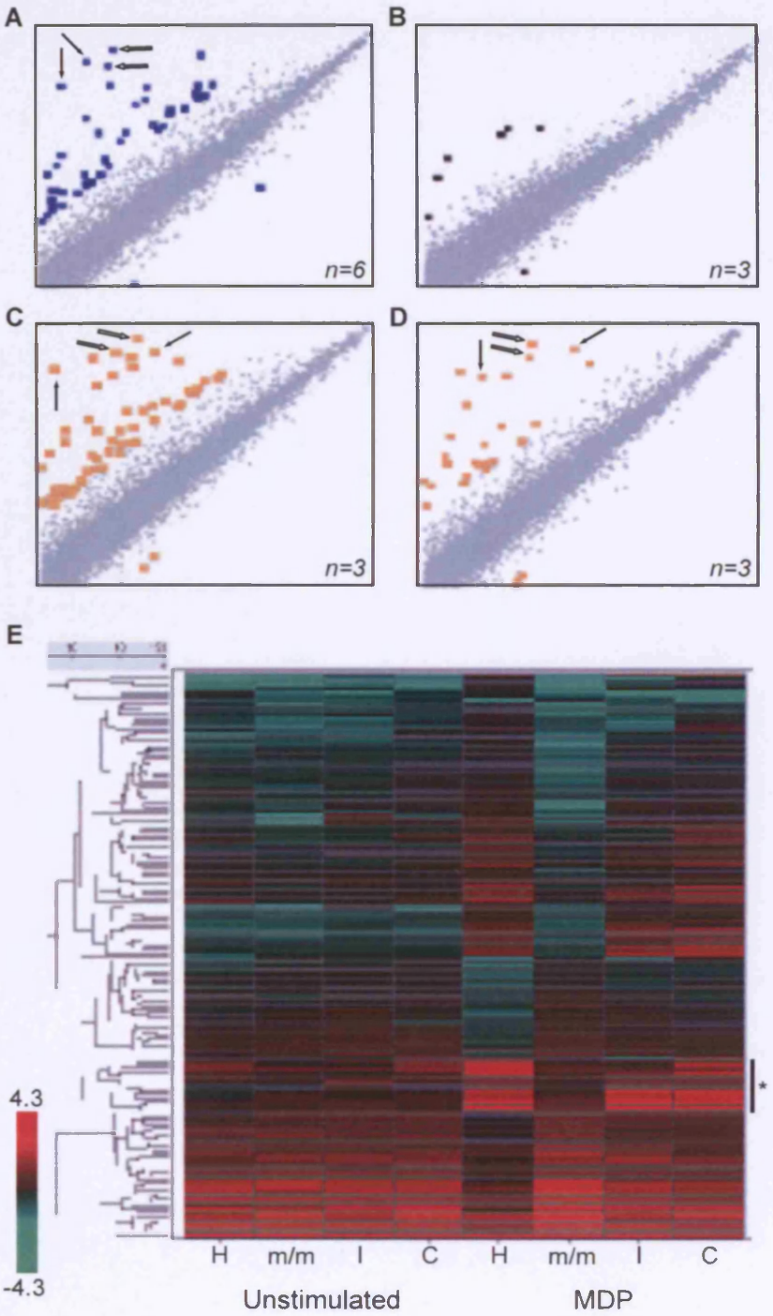


Figure 5.3 Gene expression profiles in macrophages: effects of MDP. RNA from 6 healthy subjects **(A)**, 3 *m/m* Crohn's patients **(B)** and 6 *w/w* Crohn's patients with ileal disease **(C)** or colonic disease **(D)** was analyzed using Affymetrix Human Genome U133A arrays. Transcripts for IL-8 (filled arrows) and IL-1β (open arrows) are identified except in *m/m* macrophages, which failed to respond. **(E)** Hierarchical clustering clearly illustrates a normal pattern of expression changes in *w/w* macrophages in response to MDP, which is abrogated by the *CARD15* polymorphisms. Genes of interest that were strongly induced are highlighted (*).

H: healthy control; m/m: CARD15 compound heterozygote/homozygote Crohn's patients; w/w: wild type I: w/w ileal Crohn's patients; C: w/w colonic Crohn's patients; MDP: muramyl dipeptide

Gene	HC			CD *m/m*		
	Fold Increase	SEM	*P*value	Fold Increase	SEM	*P*value
MIP-3α	6.2	0.49	*	0.87	0.3	ns
IL-8	5.8	1.17	**	1.6	0.29	ns
GRO-3	5.1	0.82	*	1.1	0.14	ns
IDO	4.7	2.23	*	1.6	0.21	ns
TSG-6	4.6	0.86	**	1.2	0.09	ns
MIP-1δ	4.5	0.27	***	1.7	1.02	ns
IL-1β	4.2	0.2	**	1.6	0.4	ns
IL-1α	3.2	0.47	*	1.4	0.39	ns
PDE-4B	2.4	0.32	**	1	0.07	ns
CD38	2.4	0.62	*	1.4	0.39	ns

Table 5.1 Genes showing the most substantial induction in macrophages from healthy controls stimulated with MDP. Corresponding fold increases in cells from CD *m/m* patients are also shown.
HC: healthy controls; CD m/m: Crohn's patients compound heterozygous/homozygous for CARD15 polymorphisms
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns: non-significant*

5.2.3 CARD15 polymorphisms abrogate the MDP gene expression response

In contrast to healthy control macrophages, cells from *m/m* Crohn's patients showed a general failure of the transcriptional response to MDP (Fig. 5.3B, Table 5.1 and Appendix 2). Only 3 probes appeared differentially expressed: these encoded ESTs of no known function or clearly recognizable homology. Their considerable inter-individual variability rendered these changes non-significant.

Macrophages from *w/w* Crohn's patients, subdivided into those with exclusively ileal (Fig. 5.3C) or colonic (Fig. 5.3D) disease, showed profiles comparable to healthy controls. Hierarchical clustering based on expression changes across the 4 groups of subjects identified a normal pattern of transcriptional responses, equivalent in all *w/w* individuals regardless of disease status; these were absent in *m/m* macrophages (Fig. 5.3E). There were no significant differences in expression profiles of unstimulated cells.

5.2.4 Post-genomic verification

To confirm that changes in gene expression were translated into functional differences in protein production, cytokine secretion by cultured macrophages incubated with MDP was measured. In concordance with the array data, concentrations of IL-1β (Fig. 5.4A) and IL-8 (Fig. 5.4B) were substantially augmented in all *w/w* but not *m/m* cells; IL-8 was elevated in a dose-dependent manner (Fig. 5.4C). Only minimal, non-significant changes were observed in IL-10 production (Fig. 5.4D) and no IL-12p70 was detected in any sample. At variance with the array data, TNF-α secreted appeared increased in *w/w* macrophages (Fig. 5.4E). This is consistent with previous work suggesting its

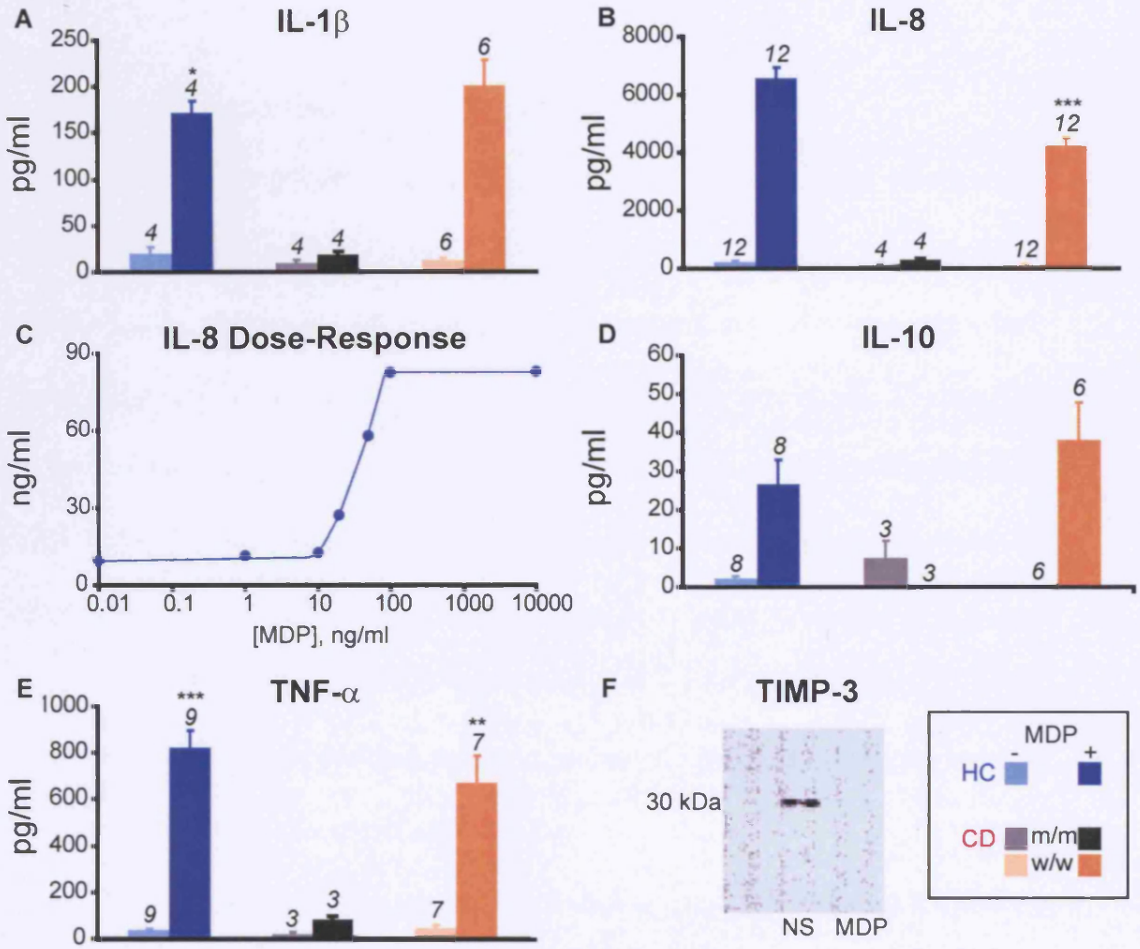


Figure 5.4 Post-genomic verification of microarray data. MDP increased the secretion of **(A)** IL-1β, **(B)** IL-8 and **(E)** TNF-α in macrophages from healthy controls and *w/w* Crohn's patients, but not *m/m* Crohn's patients. **(C)** In buffy coat macrophages, MDP induced IL-8 secretion in a dose-dependent manner (*n=3*). **(D)** Only small, non-significant changes were observed in IL-10 secretion. **(F)** Representative immunoblot confirming reduced expression of TIMP-3 in MDP-stimulated macrophages from healthy controls. Means + SEM, numbers of subjects and significance values compared to unstimulated cells are shown

HC: healthy controls; CD: Crohn's patients; NS: normal saline; MDP: muramyl dipeptide; m/m: CARD15 compound heterozygote/homozygote; w/w: wild type

** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$*

regulation by MDP at the translational rather than transcriptional level[441]. Protein levels of TIMP-3 were also studied in *w/w* macrophages, to assess a molecule with suggested transcriptional repression: MDP abolished its production (Fig. 5.4F).

5.2.5 Structural modelling of the CARD15 leucine-rich repeat

To gain further insight into the normal function of CARD15 and mechanisms underlying the effects of disease-associated polymorphisms, the predicted structure of its canonical LRR in which the polymorphisms occur was computed. Since its crystal structure remains unsolved, the model was based substantially on well-studied LRRs of other proteins such as ribonuclease inhibitor[442]. The CARD15 LRR was predicted to adopt a horseshoe conformation with a binding interface of at least 25 Å (Fig. 5.5A). In other proteins with similar motifs, the inner concave surface provides an interaction surface for binding partner proteins. The interface predicted here is considerably larger than that required to bind MDP, a small molecule of only 493 Da.

The effects on protein structure and surface charge introduced by SNP12 and SNP13 were subsequently modelled. It was not possible to model SNP8, as this occurs in a region of poorly defined structure between the LRR and NBD. SNP12 is a missense substitution that replaces a glycine residue with an arginine, introducing a positive charge on the concave inner surface of the horseshoe (Fig. 5.5B). This is likely to impede binding of any partner protein by interference with van der Waal forces. The charge alteration also provokes similar abnormalities at other points on the protein surface, which could similarly affect protein conformation and interactions with other molecules binding at these

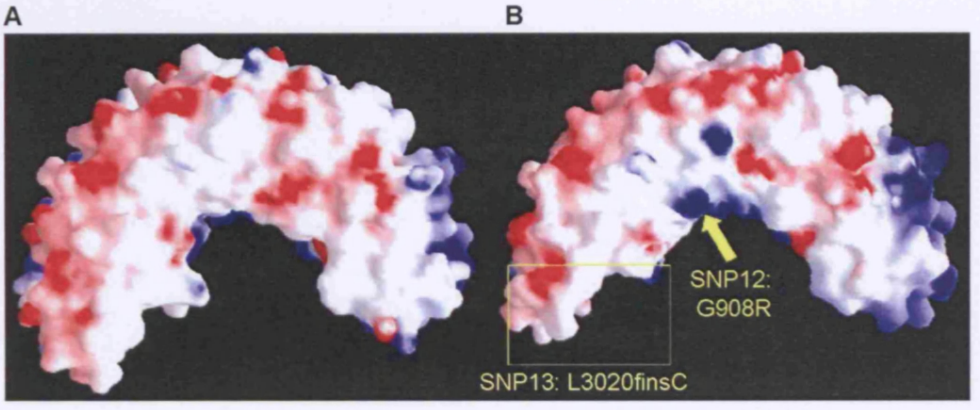


Figure 5.5 Predicted structural model of the CARD15 leucine-rich repeat. **(A)** Wild type protein. **(B)** Effects of the polymorphisms associated with Crohn's disease: SNP12 alters the surface charge and SNP13 truncates the horseshoe structure.

sites. This could prove of major importance, as many CARD-containing proteins act only when assembled into large multi-molecular complexes[284,301].

SNP13 introduces an additional cytosine nucleotide; the subsequent coding base-pair shift generates a premature STOP codon. The resultant truncated protein lacks its final α-helical loop, reducing the diameter of the predicted horseshoe binding surface (Fig. 5.5B). This also is likely to affect receptor-ligand interactions.

5.2.6 Wound fluid

CARD15 polymorphisms did not appear to explain the basal defects in neutrophil recruitment and cytokine production in the serial biopsy and skin window models. Consequently, an alternative explanation was sought. The skin window was modelled *in vitro* by exposing cultured macrophages to fluid taken from the site of acute tissue injury. Wound fluid was derived from healthy individuals having routine surgery for inguinal hernia repair. Blood and tissue fluid were recovered from the operation site 2 min after the initial incision, and cellular components removed by high-speed centrifugation.

Protein concentration of wound fluid was determined by Bradford assay. Two samples were analyzed by 2D gel electrophoresis (Fig. 5.6A), and the protein profiles found comparable to normal human serum (Fig. 5.6B). No IL-8 (as measured by ELISA) was detectable in wound fluid itself, although the latter elicited substantial, dose-dependent IL-8 secretion from buffy coat macrophages (Fig. 5.6C).

Macrophages were cultured from 55 healthy controls, 50 Crohn's patients and 20 control patients with ulcerative colitis, and the IL-8 response to wound

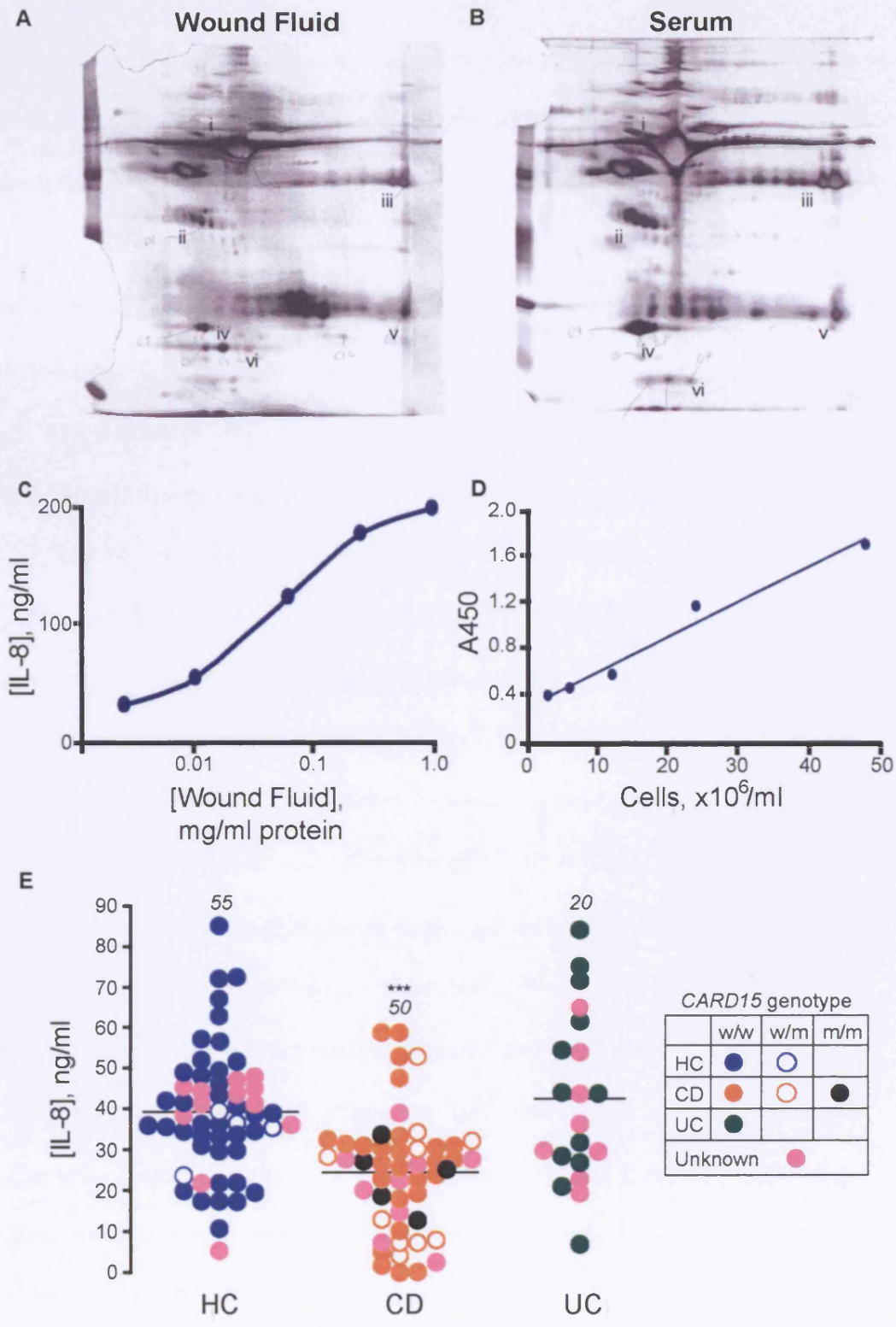


Figure 5.6 Effects of wound fluid on cultured macrophages. Protein profiles of **(A)** wound fluid and **(B)** normal human serum were similar. Spots correspond to (i) albumin, (ii) haptoglobin, (iii) IgG heavy chain, (iv) apolipoproteins, (v) IgG kappa light chain and (vi) peroxiredoxin-2. **(C)** Wound fluid elicited IL-8 secretion from buffy coat macrophages in a dose-dependent manner ($n=3$). **(D)** Validation of the WST-8 assay, illustrating that A450 varies linearly with cell numbers in the range used in these experiments ($n = 3$). **(E)** Reduced secretion of IL-8 by CD macrophages in response to wound fluid, irrespective of *CARD15* genotype. Means, numbers of subjects and significance compared to HC shown.
HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients
*** $P < 0.001$

fluid determined. Secretion was normalised to numbers of viable cells using the WST-8 assay based on the activity of intracellular dehydrogenases[373]; a linear relationship with cell numbers was demonstrated (Fig. 5.6D). In control subjects, high concentrations of IL-8 (comparable to those observed in skin windows) were secreted, with a normal distribution possessing high and low responders at each tail (Fig. 5.6E). In Crohn's patients, the distribution was shifted towards lower concentrations, with a significant reduction in mean production ($P < 0.001$). Secretion was not related to *CARD15* genotype in any group.

Wound fluid contains numerous pro-inflammatory mediators. In an attempt to identify those eliciting a weak response in Crohn's disease, several basic characterisations were performed. Wound fluid-induced IL-8 production by healthy macrophages (n = 5) could be modestly inhibited by the G-protein inhibitors cholera toxin (for G_s[443]; $P < 0.05$) or pertussis toxin (for $G_{i/o}$[444]; $P < 0.05$), and by the thrombin inhibitor PPACK[445] ($P < 0.05$). A more dramatic inhibition (approximately 50%) was achieved using broad spectrum protease inhibitors ($P = 0.004$) or pre-heating the fluid to 56°C (Fig. 5.7A,B; $P = 0.004$). None of these stimuli were detrimental for cell viability.

Protease inhibitors and preheating were assessed for differential effects on Crohn's macrophages (Fig. 5.7B). Whereas protease inhibitors resulted in similar absolute reductions in IL-8 secretion in both groups, the absolute reduction induced by pre-heating was attenuated in Crohn's patients. This indicates that there is a heat-labile component to which Crohn's patients are less sensitive. Although a number of enzymes are denatured on heating, another mediator classically inhibited under these conditions is complement[446]. This would be expected to be present in wound fluid and complement-induced lysis of

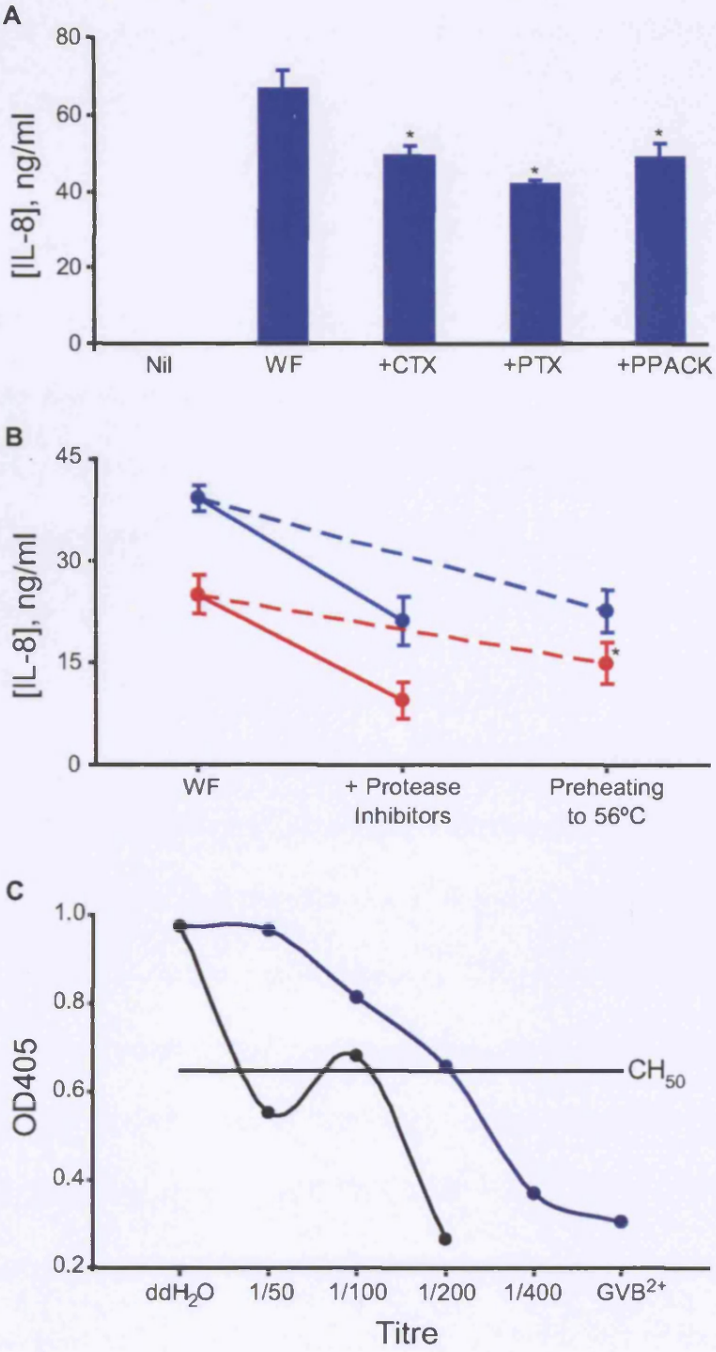


Figure 5.7 Further characterisation of wound fluid. **(A)** Effects of various inhibitors on the ability of wound fluid to stimulate IL-8 secretion from buffy coat macrophages. Means + SEM and significance compared to wound fluid alone are shown ($n = 5$ for each condition). **(B)** Treatment of wound fluid with protease inhibitors or preheating reduces its potency; sensitivity to latter is diminished in Crohn's (red) compared to healthy (blue) macrophages ($n = 5$ for both). Significance value refers to absolute difference compared to that induced by protease inhibitors. **(C)** Lysis of sheep erythrocytes induced by wound fluid (black line) compared to normal human serum (blue line), indicating complement activity ($n = 3$ for both).

WF: wound fluid; CTX: cholera toxin; PTX: pertussis toxin; GVB: gelatin veronal buffer

** $P < 0.05$*

sheep erythrocytes was demonstrable (n = 3), with a CH_{50} at an approximate titre of 1:100 (Fig. 5.7C).

5.2.7 C5a, TNF-α and LPS

To directly assess the response to complement, macrophages were stimulated with recombinant human complement component C5a. In healthy controls and ulcerative colitis patients, C5a elicited IL-8 secretion; this was significantly diminished in Crohn's patients (Fig. 5.8A; $P < 0.001$). Although there was an overlap between the groups, 50% of Crohn's patients exhibited IL-8 secretions below the 10^{th} centile of healthy controls.

Macrophages were then exposed to other inflammatory agonists to determine the stimulus specificity of the defect. A similar pattern of reduced IL-8 secretion was observed following exposure to TNF-α (Fig. 5.8B; $P < 0.001$), but not LPS (Fig. 5.8C). A shared defect between C5a and TNF-α suggests a molecular lesion downstream of their receptors, but above the IL-8 gene given the normal response to LPS. This is supported by the correlation in Crohn's macrophages of responses to C5a and TNF-α ($R = 0.55$, $P < 0.01$), but not between either stimulus and LPS ($R = 0.378$, non-significant). Responses to each agonist were unrelated to *CARD15* genotype (Fig. 5.8A-C), site of disease (Fig. 5.9A) and use of medication (Fig. 5.9B). All patients had quiescent disease.

5.2.8 Phosphorylation of p44/p42 MAP kinase in response to C5a

The stimulus-selective failure of IL-8 production implicates a probable abnormality in cytoplasmic signalling pathways or the transcriptional apparatus. The signal transducer p44/p42 MAP kinase is known act downstream of the C5a

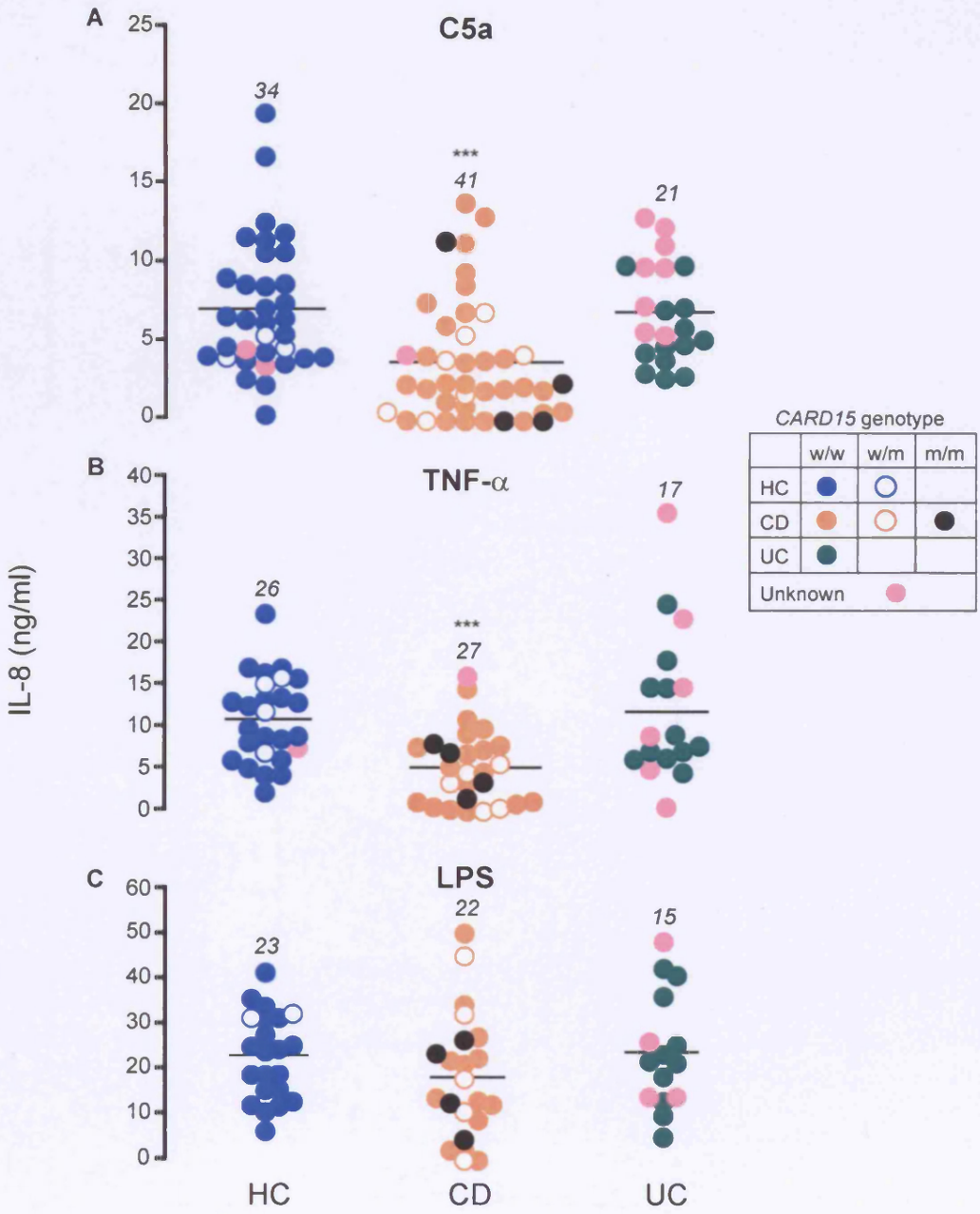


Figure 5.8 Response to C5a, TNF-α and LPS. Secretion of IL-8 by HC, CD and UC macrophages in culture following stimulation with **(A)** C5a, **(B)** TNF-α or **(C)** LPS for 6 h. Response was independent of *CARD15* genotype. Mean values and numbers studied are shown.
HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients
*** $P < 0.001$

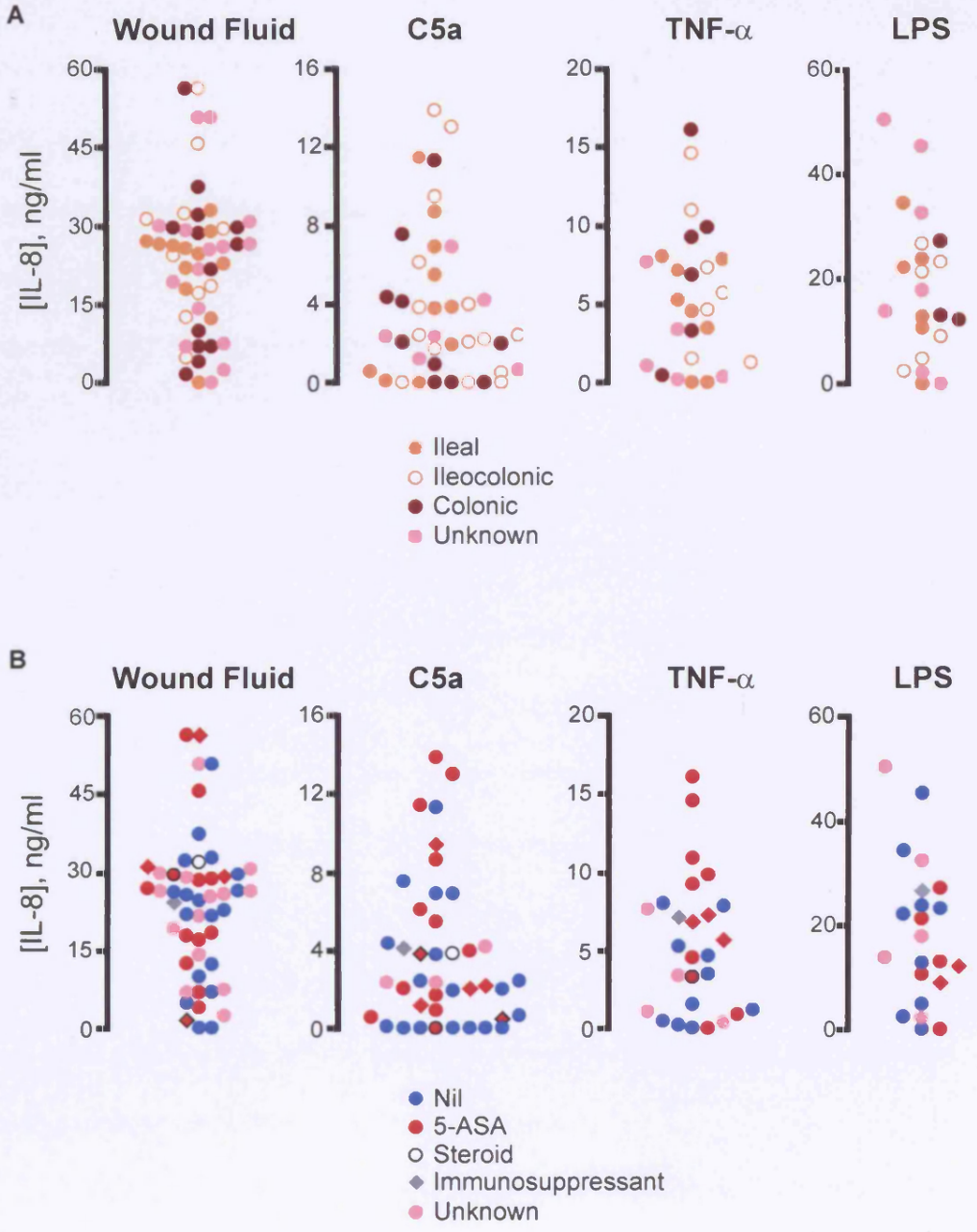


Figure 5.9 Subject characteristics in macrophage culture studies. IL-8 responses in Crohn's patients to the different inflammatory agonists did not vary according to **(A)** disease location or **(B)** use of medication.

receptor[447], and becomes phosphorylated in association with IL-8 production[448]. In buffy coat macrophages, it was minimally phosphorylated when unstimulated, with rapid phosphorylation (maximal at 2 min) following exposure to C5a (Fig. 5.10A). Its phosphorylation state under these conditions was assessed in a small number of subjects (Fig. 5.10B), but no difference apparent between high (n = 5) and low (n = 5) responding Crohn's patients and healthy controls (n = 7). Interestingly, in the 3 ulcerative colitis patients studied in this experiment, all appeared to show hyperphosphorylation.

5.3 Discussion

5.3.1 Response to MDP

Polymorphisms in *CARD15* have been proposed to play a central role in the pathogenesis of Crohn's disease[264,449]. The mechanisms of the predisposition, as well as the normal functions of the protein, remain poorly understood. Under basal conditions, predominant *CARD15* expression occurs in mononuclear phagocytes[279,280]. Originally thought to act as a LPS receptor, it was subsequently demonstrated to respond instead to MDP[288,289]. Detection of this bacterial product normally leads to NF-κB activation, which is abrogated in the presence of polymorphisms associated with Crohn's disease.

In this study, *CARD15* polymorphisms did not influence IL-12 secretion by macrophages in response to LPS, further refuting the initial data[265]. The response to MDP was subsequently examined. At the time these experiments were conducted, its effects on macrophages and appropriate outcome measures were unknown. Due to cost considerations, the stimulus duration and concentration applied were based on those published in the literature[288] which

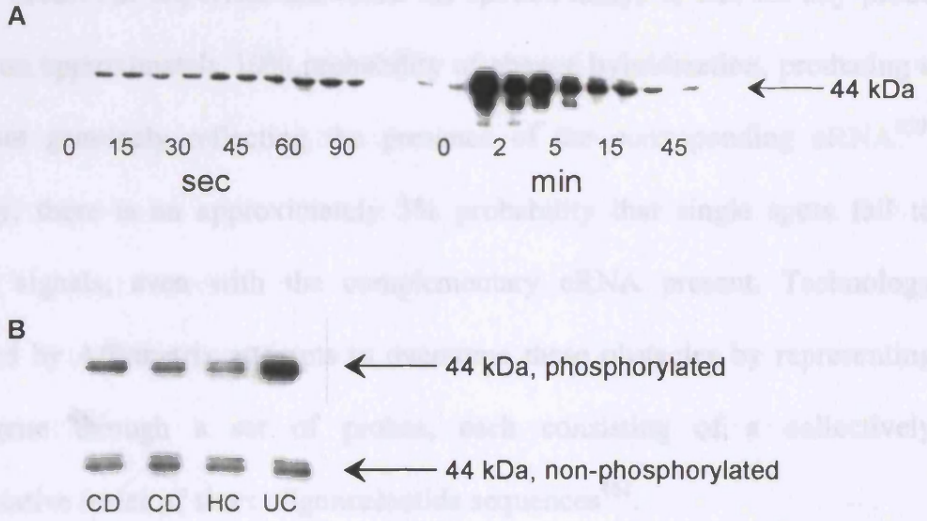


Figure 5.10 Phosphorylation of p44/p42 MAP kinase in macrophages stimulated with C5a. **(A)** Immunoblots showing the time course of changes in buffy coat macrophages. **(B)** Representative immunoblot illustrating normal phosphorylation in 2 CD patients (1 high responder, 1 low responder in IL-8 assays), 1 HC subject and 1 UC patient. The latter demonstrates the hyperphosphorylation observed in all UC patients studied.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients

also corresponded well to the conditions employed in the *in vivo* studies performed here (see *4.2.6*).

DNA microarrays represent a powerful methodology for determining global changes in gene expression, particularly useful when the outcome of exposure to a stimulus is unknown beforehand. In addition, they provide considerable contextual information about the background state of cells in which changes occur. An important drawback for spotted arrays is that for any probe there is an approximately 10% probability of chance hybridization, producing a signal not genuinely reflecting the presence of the corresponding cRNA[450]. Similarly, there is an approximately 5% probability that single spots fail to produce signals, even with the complementary cRNA present. Technology employed by Affymetrix attempts to overcome these obstacles by representing every gene through a set of probes, each consisting of a collectively representative series of short oligonucleotide sequences[451].

The cRNA data were processed by subtracting background fluorescence values for each array from those of the individual spots. Multiple arrays were compared by normalization to equivalent levels of total fluorescent intensity, usually achieved as here by dividing values for each signal by the signal mean over the whole array[452] followed by log transformation. Processing data in this manner also helps overcome the problem that distributions of raw expression values tend to be skewed, violating the normality assumption required for most statistical tests.

Differences in expression levels are usually analyzed in terms of fold change between the various conditions employed, with 2-fold alterations considered the threshold for significance[453]. This has been criticized because of

poor signal-to-noise ratios for genes with low expression and impaired sensitivity for true positives that are highly expressed originally. To increase confidence in the results of this study, significance of fold changes were confirmed by 2-way ANOVA on all genes of interest[454]. Differences in transcripts of prime interest were also validated using post-genomic techniques, to verify their functional importance.

The genes principally induced in macrophages by MDP were pro-inflammatory. They included a number of chemotactic cytokines of which IL-8 and IL-1β were prominent members, corresponding closely with the skin window studies (see *4.2.6*). Abrogation of the transcriptional response, and identical basal gene expression profiles, in *m/m* macrophages argues in favour of loss of the pro-inflammatory response rather than a hyper-responsive gain-of-function caused by *CARD15* polymorphisms. The normal response of macrophages from *w/w* Crohn's patients suggests that these individuals are unlikely to possess molecular lesions elsewhere along the same signalling pathway. It would be interesting to repeat these studies in both a large population of simple heterozygotes (to determine whether they possess an intermediate phenotype) and in *m/m* healthy controls (in whom the response should be abrogated). Although one such individual was identified in this study, it was not possible to obtain a further blood sample for this experiment.

Mechanistically important changes were confirmed to be translated into protein effects. As predicted, IL-8 and IL-1β secreted were considerably enhanced. IL-10 was examined as a cytokine for which only small, non-significant elevations in mRNA levels were implicated by the array; this was borne out by the concentrations secreted. No transcripts sufficient to produce the

IL-12p70 heterodimer were detected in any sample, and the cytokine was not present in the culture supernatants. The only cytokine measured that demonstrated a poor correlation was TNF-α, for which mRNA levels were rather variable across the samples with no clear pattern of change induced by MDP. In contrast, its secreted concentration was clearly augmented following stimulation. This is consistent with previous reports suggesting that this cytokine is principally regulated at the post-translational stage[441], largely by controlling cleavage of the pro-cytokine by TNF-α-converting enzyme (TACE) in the plasma membrane[455].

The transcriptional profiles observed here correspond favourably with those subsequently reported in the literature[403]. Both contrast with other reports suggesting MDP action through CARD15 elicits production of anti-inflammatory IL-10[292], and that loss of negative regulation in *m/m* individuals predisposes to chronic inflammation. These apparently polarized findings are not mutually exclusive, since the latter study stimulated mononuclear cells over a longer 48 h time course. It is clearly documented that many pro-inflammatory agonists, including LPS, initiate counter-regulatory mechanisms in addition to those that drive inflammation to limit its duration[456]. The question then arises as to which phase of the response contributes to the pathogenesis of Crohn's disease. Loss of anti-inflammatory regulation should manifest in a model such as the skin window with normal initiation of inflammation but prolonged resolution. Conversely, the reduced accumulation of neutrophils and cytokine production observed (see Chapter 4) supports a key role for pro-inflammatory mediator deficiency.

5.3.2 CARD15 may not bind MDP directly

A final consideration raised by this study pertains to whether CARD15 directly binds MDP, as currently proposed. Although the LRR domain typically mediates protein-ligand interactions, the predicted binding surface is considerably larger than required. One possibility would be presentation of MDP by a chaperone protein. This could explain the interaction with CARD15, which resides in the cytoplasm[279]. It is not otherwise apparent how MDP crosses the plasma membrane, although it has been proposed that CARD15 does moves to this cellular compartment on activation[457]. A final possibility would be a specific membrane receptor for MDP that initiates an intracellular signalling cascade activating CARD15 downstream.

Should MDP bind CARD15 directly, the interaction need not occur at the concave surface. CARD15 bears significant structural homology to R proteins[458] that mediate the immune or "hypersensitive" response in plants[459]; these also possess LRRs. Whilst originally believed to bind microbial avirulence products directly[460], this has never been satisfactorily demonstrated. A more recent theory, known as the guard hypothesis[461], suggests that R protein LRRs bind instead to normal cellular proteins (the virulence targets). Detection of any alteration in their targets induces a rapid defence response. Whatever binding partner associates with the concave aspect of the CARD15 LRR, computer modelling here suggests that SNPs associated with Crohn's disease act by disturbing this interaction. MDP could fit into the equation by associating with either another surface of the CARD15 molecule or an alternative protein to which it is complexed.

5.3.3 Alternative inflammatory agonists

Polymorphisms in *CARD15* do not appear to explain the generic impairment in cytokine production or neutrophil recruitment in Crohn's disease. In an attempt to understand the cellular basis of this failure, macrophages were studied in culture to model cytokine production in the skin window. The novel stimulus of wound fluid, taken from an acute surgical incision, was used to reproduce a physiological mixture of inflammatory stimuli similar to that present at the site of epithelial trauma induced by skin abrasion. A stimulus of this nature has never been used previously to stimulate macrophages.

In control subjects, substantial IL-8 production was elicited in concentrations reminiscent of those measured in skin windows. Secretion in response to this general agonist was diminished in Crohn's macrophages, supporting this as a good *in vitro* model of the skin window. The intrinsic failure of IL-8 production by these cells further argues against the defect in neutrophil migration relating to a circulating inhibitor of inflammation[235].

A number of pro-inflammatory mediators will be present in wound fluid; extensive characterisation was beyond the scope of this study. A number of general observations were made: the protein content appeared similar to normal human serum, and activated complement was demonstrable by lysis of opsonized sheep erythrocytes[446]. Although protease inhibitors substantially attenuated stimulation of IL-8 secretion, this was equivalent in healthy and Crohn's macrophages. In contrast, the latter showed a restricted diminution after preheating wound fluid, suggesting reduced sensitivity to its heat-labile fraction.

Since complement is characteristically inactivated by heat treatment, pure recombinant C5a was assessed as an agonist and the response found defective in

a significant proportion of Crohn's patients. The defect appeared to be shared with TNF-α (and may extend to other untested stimuli) but not LPS, implicating an abnormality in the cell signalling or transcriptional apparatus. The lesion could not be identified in the preliminary studies described here: the phosphorylation of p44/p42 MAP kinase, known to act downstream of the C5a receptor and associated with IL-8 production, was normal. The exception was in ulcerative colitis patients, in whom it appeared hyperphosphorylated after stimulation. Although based on a small sample, this may be worth validating and could prove informative about the pathogenesis of this disease.

A failure in the response to complement is noteworthy given the predisposition of Crohn's inflammation for the lower gastrointestinal tract. The lumen contains considerable numbers of bacteria, which can ingress into the bowel wall through any breach in the mucosal barrier. Bacterial cell walls activate the alternative pathway of complement[462], and gut bacteria such as *Escherichia coli* strongly trigger C5a production[463]. Complement could be critical for triggering homeostatic inflammation to remove bacterial debris from the gut, since the principal repertoire of pathogen recognition receptors (CD14 and TLRs) are normally down-regulated on intestinal macrophages and enterocytes[42,87]. This is usually beneficial, preventing excessive immune activation in non-pathogenic conditions, but how then to sense potentially deleterious infection? The C5a receptor by comparison is expressed at high levels on intestinal macrophages[464]. One possibility is that when bacterial loads in the bowel wall become sufficient to cause serum extravasation, activation of the complement cascade through the alternative pathway initiates a local inflammatory response.

Interestingly, some congenital complement deficiencies have been associated with enteropathies first diagnosed as Crohn's disease[465,466], and C5-deficient mice exhibit more prolonged and severe inflammation in DSS colitis[467]. An impaired chemotactic response of Crohn's neutrophils to C5a has also been reported[468], which could indicate a generic problem in signalling pathways downstream of its receptor.

These experiments highlight the heterogeneity inherent in Crohn's disease, supporting the hypothesis that it actually represents a syndrome with a variety of molecular causes producing a final common phenotype[469]. As such, alternative components of wound fluid (which could be investigated using chromatographic or filtration-based methods), cellular aberrations or molecular lesions might underlie failure of cytokine production or acute inflammation in different Crohn's patients. This would be consistent with a failure as suggested here of the LPS response in a minority of patients (*5.2.1* and possibly 4 patients in *5.2.7*). Given the previously documented mutations in components of the LPS-sensing pathway in Crohn's disease, it would be interesting to sequence the relevant genes (*LBP*[318], *CD14*[319], *TLR4*[470], *MD-2*[321] and *MyD88*[471]) in the non-responders identified in this study.

The data presented in this chapter add further support to the concept of a general systemic defect in innate immunity in Crohn's disease. Again, it is of interest that the abnormalities appear entirely unrelated to *CARD15* genotype, questioning the centrality previously afforded to it as a primary pathogenic lesion. The hypothesis that the CARD15 pathway provides a compensatory mechanism to augment acute inflammation was elaborated previously (see *4.3.4*). Such secondary processes may determine the principal sites of inflammation, as

CARD15 does for ileal disease, based on the nature of the microbial sensing defect and the contents of the luminal flora.

5.3.4 Functional relevance of diminished acute inflammation

Despite the probable multiplicity of underlying molecular mechanisms, the general unifying feature in Crohn's patients appears to be a predisposition towards a weak acute inflammatory response. This could potentially culminate in suboptimal clearance of bacteria invading the tissues and a granulomatous reaction to prevent collateral dissemination of infective material. The crux of this hypothesis is that Crohn's patients will exhibit weak responses to the presence of bacterial or other foreign material in the tissues. To assess this directly, the *in vivo* reaction to subcutaneous inoculation with heat-killed *Escherichia coli* was examined (Chapter 6).

Chapter 6: Bacterial Injections

6.1 Introduction

Data from the previous chapters demonstrated that Crohn's patients possess a general impairment in their inflammatory response, manifested by reduced neutrophil recruitment to sites of epithelial damage (see Chapters 3 and 4). The functional consequences of this abnormality *in vivo* remain undetermined. Bacteria and their products will ingress through any discontinuity in the mucosal barrier[36]. The hypothesis generated by this thesis predicts that any inflammatory response generated by their introduction into the tissues will be weak. Delayed clearance could then drive chronic inflammation.

Escherichia coli are gram-negative aerobic bacilli prevalent in the normal bowel flora[472], which colonize the gastrointestinal tract within hours of birth. The relationship between host and microbe is usually symbiotic, with the exception of a few pathogenic strains. Commensal strains rarely cause disease, apart from in the immunocompromised host or following disruption of the normal mucosal barrier[473]. In the event of the latter, they constitute a major population of bacteria infiltrating the bowel wall. A failure to clear such organisms is postulated in Crohn's disease, given the reduced neutrophil chemotaxis. Consistent with this prediction *Escherichia coli* DNA has been detected within macrophages of Crohn's granulomata[474]. Whilst apparently supporting the hypothesis, there are inherent difficulties in drawing inferences from chronic established lesions. Consequently, the *in vivo* acute inflammatory response of Crohn's patients following introduction of killed *Escherichia coli* into the tissues was examined.

6.2 Results

6.2.1 Bacterial preparation

An antibiotic-sensitive clinical isolate of *Escherichia coli* NCTC 10418 was grown up in synthetic medium, killed by heating and divided into aliquots of 1.05 mg dry weight. Five randomly selected aliquots were cultured by UCLH Clinical Microbiology under standard conditions and in enriched broth for 7 days at room temperature, 30°C and 37°C: no growth was observed. Aliquots were resuspended prior to use to a concentration of 5 x 10^9 bacteria/ml (see *2.6.1*), which contained 5 mg/ml protein as measured by Bradford assay.

To determine the kinetics of bacterial clearance from the forearm *in vivo*, a γ-emitting ^{99m}technetium label was attached[375]. Two labelling techniques were assessed, with radioisotope incubation in the presence of stannous pyrophosphate or lipophilic sodium exametazime[475]. The latter had three-fold greater labelling efficiency, and was more stable following 6 h *in vitro* incubation of labelled bacteria with serum or neutrophils (Table 6.1).

A healthy individual was inoculated with 200 µl labelled *Escherichia coli* (activity = 115,200 counts per second). A signal could be detected at the forearm injection sites over the subsequent 24 h (Fig. 6.1A). Peripheral venous blood taken at 6 h contained 30 counts per second (urinary excretion was not determined). Technetium possess a 6 h half-life[476] and a 70 kg male has a total blood volume of approximately 5 litres. This equates to approximately 41,000 counts per second of released radiolabel in the circulating blood volume, or 36% of that attached to the bacteria when injected. Visualization of label in the forearm at 48 h using a γ-camera also demonstrated that undigested material

Experiment	Label	Pellet	Supernatant
Labelling Efficiency	Stannous pyrophosphate	3 MBq	16 MBq
	Lipophilic exametazime	7 MBq	12 MBq
Stability (6 h Incubation)	Stannous + Neutrophils	180 cps	80 cps
	Stannous + Serum	150 cps	200 cps
	Lipophilic + Neutrophils	2,000 cps	20 cps
	Lipophilic + Serum	1,000 cps	60 cps

Table 6.1 Efficiency and stability of *Escherichia coli* radiolabelling with two different techniques.

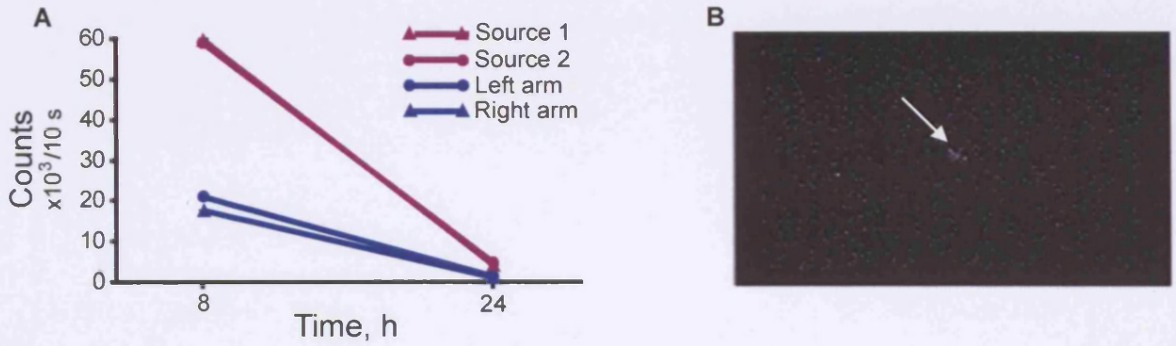


Figure 6.1 Detection of radiolabelled bacteria injected into the forearm of a healthy control. **(A)** Signal could be readily measured over the subsequent 24 h using a gamma counter. Comparison to source and peripheral blood activities would allow quantification of bacterial clearance in future experiments. **(B)** Signal was still present at 48 h, visualized with a gamma camera. These images also illustrated that bacteria remain highly localised to the inoculation sites (arrow) prior to clearance.

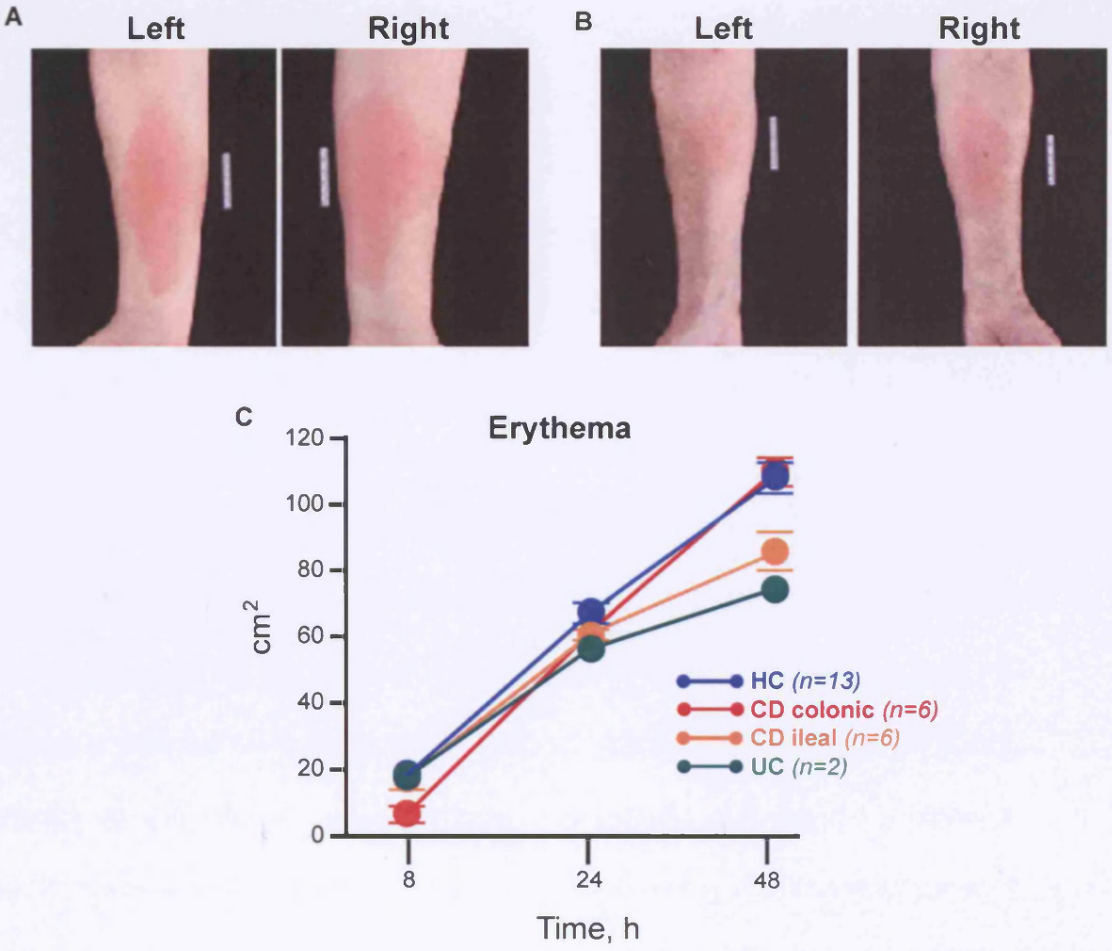


Figure 6.2 Erythematous reactions after inoculation with killed *Escherichia coli*. Erythema developed in **(A)** healthy controls and **(B)** Crohn's patients. Left and right forearms from one subject of each group are shown, scale bar = 5 cm. **(C)** Areas of erythema were not significantly different in any subject group. Results shown as mean ± SEM.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients

remained localized to the injection site over the time course of the experiment (Fig. 6.1B).

6.2.2 Acceptability

Bacterial injections were conducted on 13 healthy controls, 12 Crohn's patients and 2 patients with ulcerative colitis (Appendix 1: Tables A1.8-A1.10 respectively). All patients were in clinical remission and the majority of subjects were off medication. Given the difficulty in recruiting subjects for these experiments, a few patients were enrolled receiving treatment. These included 6 Crohn's patients on 5-aminosalicylates of whom 1 also took azathioprine and another methotrexate, and 1 ulcerative colitis patient on olsalazine.

In all subjects, injection elicited local inflammation within 2-4 h, characterized by erythema (Fig. 6.2A,B), oedema, and local pain with tenderness. Pain was maximal 12 h after inoculation, rated as 4.57 ± 2.09 (mean ± SEM) on a 10-point visual analog scale. Analgesia was required and successfully ameliorated pain in 10 participants; this included 1 g paracetamol (7 subjects), 300 mg aspirin (1 subject) and 10/500 mg co-dydramol (2 subjects).

Systemic symptoms included nausea (9 subjects), myalgia of the neck and shoulder girdle (8 subjects), headache (5 subjects), shivering/rigors (3 subjects), pyrexia (3 subjects), vomiting (2 subjects), malaise (2 subjects), lethargy (2 subjects), sweating/flushing (2 subjects), sore throat (1 subject), axillary pain without lymphadenopathy (1 subject) and gastrointestinal upset (1 subject). These were typically mild, occurred within 12 h of injection and almost completely resolved by 24 h. The profile of adverse reactions was similar across

all subject groups, except for a serious adverse event in one ulcerative colitis patient (see *6.2.10*).

6.2.3 Local response in healthy controls

In healthy individuals, the area of erythema spread progressively over the course of the experiment (Fig. 6.2A,C). After 24 h, it became more diffuse and disappeared completely by 72 h. Laser Doppler measurement of blood flow around the injection site showed substantial increases by 8 h (Fig. 6.3A,D; $P < 0.001$), maximal by 24 h (Fig. 6.3B,D; $P < 0.001$), and a return almost to baseline by 48 h (Fig. 6.3C,D). Measurements were highly correlated between left and right arms (Fig. 6.3E; $R = 0.918$, $P < 0.001$), and no changes observed in the lower limbs (monitored as a control vascular bed).

6.2.4 Impaired response in Crohn's patients

Although superficial appearances in Crohn's patients were similar (Fig. 6.2B,C), elevations in blood flow were abnormally low at 8 h (Fig. 6.4A; Fig. 6.3D) and 24 h (Fig. 6.4B; Fig. 6.3D), returning to baseline by 48 h (Fig. 6.4C; Fig. 6.3D). This was most marked in patients with colonic disease ($P < 0.001$), although those with ileal disease also showed a partial defect ($P < 0.01$). Half of the latter patients were *CARD15 m/m*; their response was indistinguishable from *w/w* Crohn's subjects. The degree of impairment was unrelated to use of medication or smoking status.

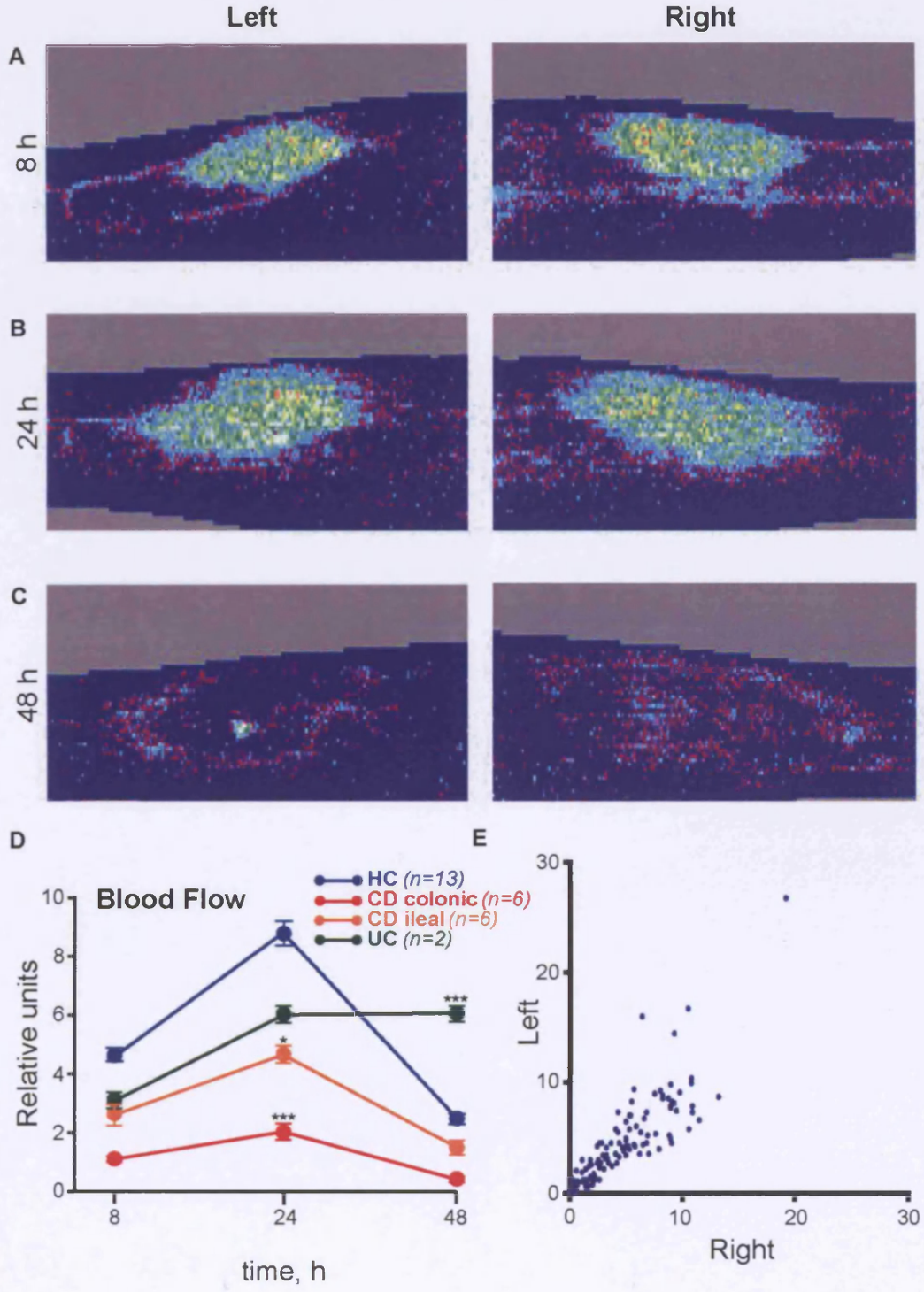


Figure 6.3 Blood flow responses following subcutaneous injection of *Escherichia coli*, measured using laser Doppler. **(A-C)** Representative laser Doppler images from the left and right arms of a representative healthy control at **(A)** 8 h, **(B)** 24 h and **(C)** 48 h. Pixel intensity linearly correlates with blood flow. **(D)** Time course of changes shows reduced blood flow in CD, particularly the colonic variety, and delayed resolution in UC. Results shown as mean ± SEM. Significance values compared to HC at same time point. **(E)** Blood flows in left and right arms were highly correlated in each subject at every time point.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients

** $P < 0.05$; *** $P < 0.001$*

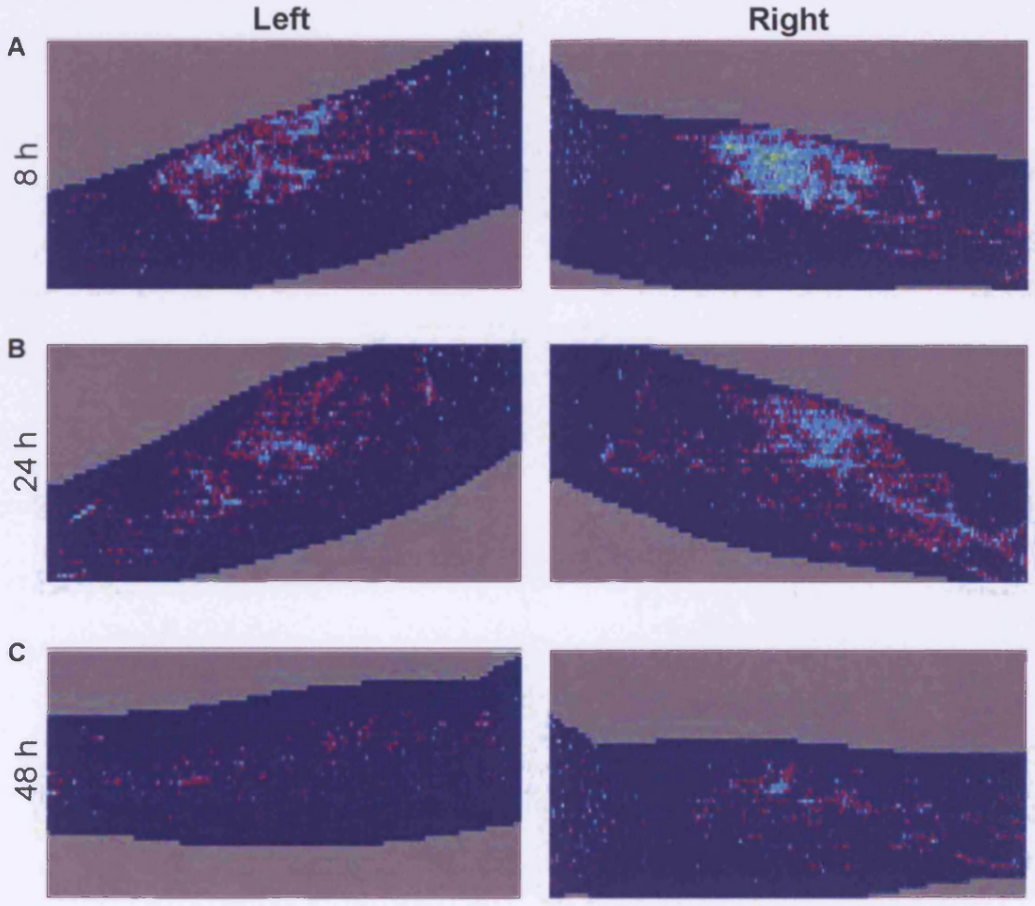


Figure 6.4 Blood flow responses in a representative colonic Crohn's patient. **(A)** 8 h, **(B)** 24 h and **(C)** 48 h following injection. Laser Doppler images for left and right arm are shown at each time point.

6.2.5 Systemic response

Bacterial injection also elicited a systemic inflammatory response, including a raised peripheral blood leukocyte count at 24 h ($P = 8.6x10^{-6}$), which returned close to baseline by 48 h (Fig. 6.5A). The 48 h levels were higher in colonic Crohn's patients than controls ($P = 0.003$), although baseline counts were also raised ($P = 0.03$). The increases in all subjects were largely due to neutrophilia ($P = 0.2x10^{-6}$), again greater in Crohn's patients (Fig. 6.5B; $P = 0.004$). Monocyte numbers were also elevated (Fig. 6.5C; $P = 0.01$), and lymphocytes reduced (Fig. 6.5D; $P = 0.04$), without significant differences between subject groups. Eosinophil (Fig. 6.5E) and basophil counts (Fig. 6.5F) were unchanged, as were other parameters of the full blood count including haemoglobin and platelet numbers.

Concentrations of multiple serum cytokines were assayed using an array-based system, at baseline, 24 h and 48 h. These included IL-1β (Fig. 6.6A), IL-6 (Fig. 6.6B), IL-8 (Fig. 6.6C), IL-10 (Fig. 6.6D), IL-12 (Fig. 6.6E), TNF-α and IFN-γ. Only IL-6 was elevated in all samples, attaining highest concentrations in colonic Crohn's patients ($P < 0.05$). TNF-α and IFN-γ were only detected in a minority of samples, with no difference between patients and controls. All induced cytokines showed peak elevation at 24 h, with return to baseline by 48 h, but only IL-6 concentrations rose to a physiologically relevant range[378,477,478]. Systemic acute phase reactants CRP (Figure 6.7A) and SAA (Figure 6.7B) were also increased by 24 h, reaching even greater levels by 48 h. Their induction was significantly higher in colonic Crohn's patients ($P < 0.01$ for each). These serum factors were undetectable or within normal limits in all subjects at baseline.

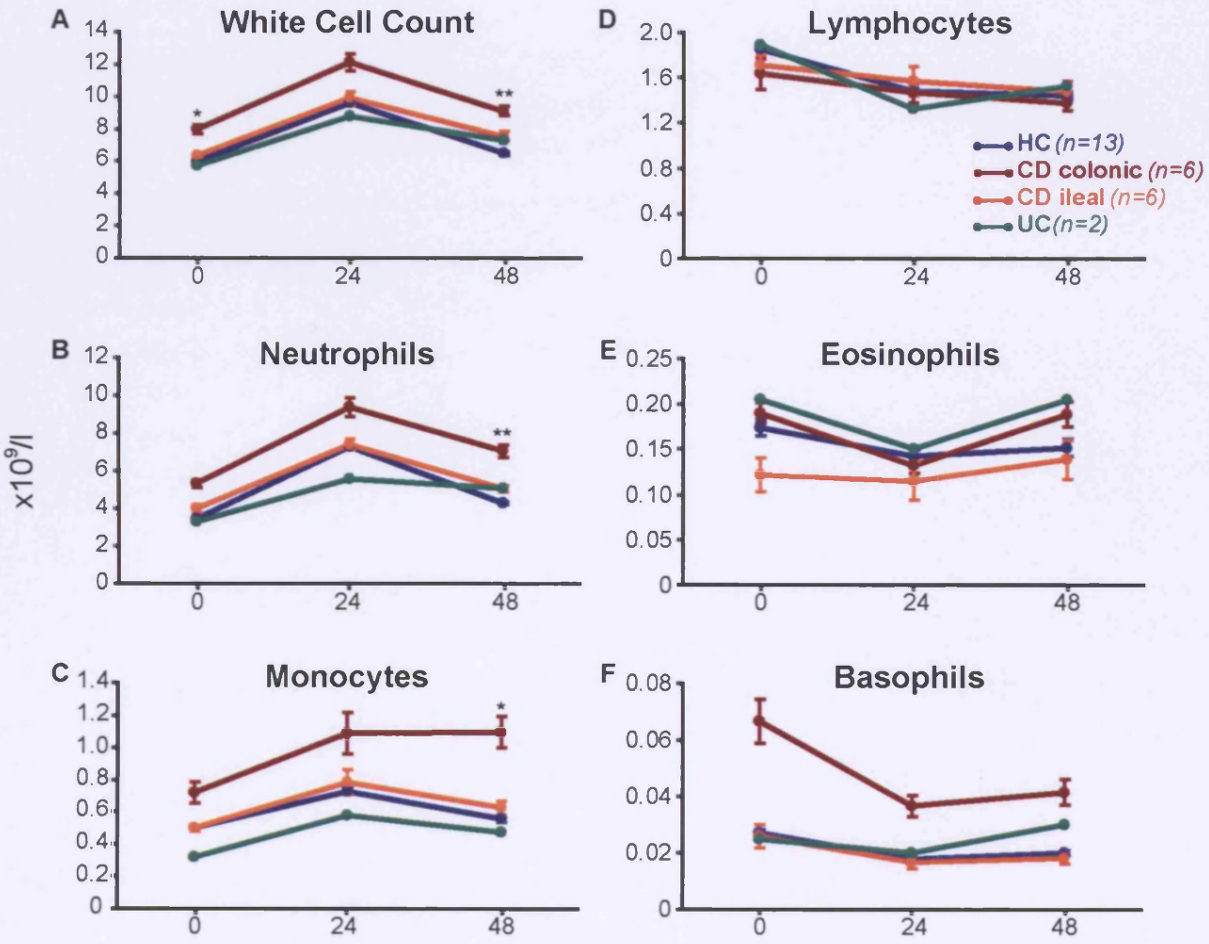


Figure 6.5 Changes in peripheral blood leukocyte counts. **(A)** Total systemic leukocyte counts following injection. **(B)** Neutrophils. **(C)** Monocytes. **(D)** Lymphocytes. **(E)** Eosinophils. **(F)** Basophils. Results shown as mean ± SEM. Significance values compared to HC at same time point.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients
*$P < 0.05$; **$P < 0.01$

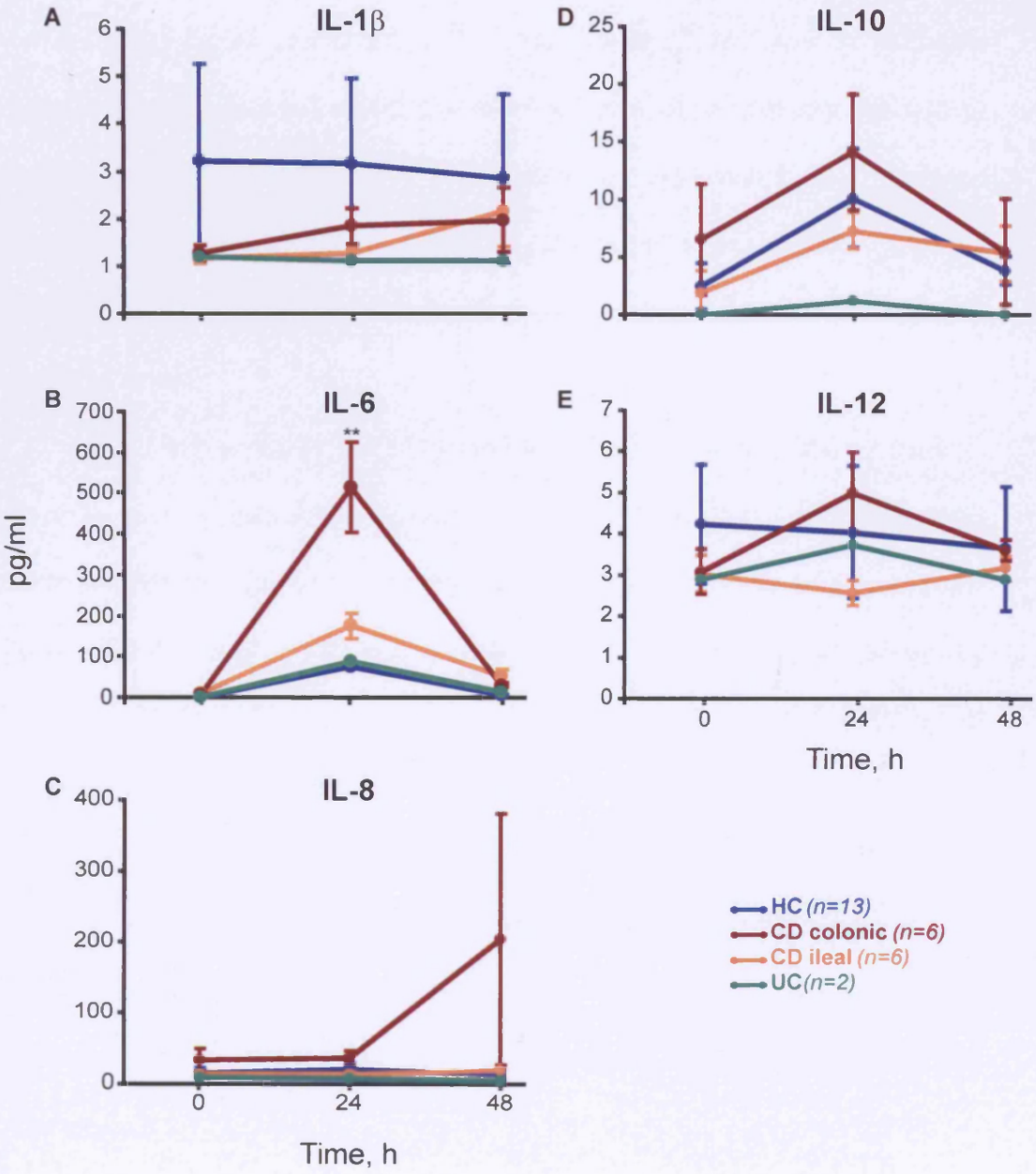


Figure 6.6 Changes in serum cytokine concentrations. **(A)** IL-1β. **(B)** IL-6. **(C)** IL-8. **(D)** IL-10. **(E)** IL-12. Results shown as mean ± SEM. Significance values compared to HC at same time point.

HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients

*** $P < 0.01$*

6.2.6 Bacterial digestion products reduce vascular tone

The presence of bacteria in the tissues normally induces neutrophil recruitment. Following their phagocytosis and digestion, the vacuole bursts releasing solubilized material extracellularly[205]. Many bacterial cell wall constituents, including LPS, can interact with the vascular endothelium directly and lead to smooth muscle relaxation[479,480]. A potential explanation for impaired vasodilatation in Crohn's disease could be reduced generation of such vasoactive products secondary to diminished neutrophil extravasation and consequently bacterial uptake.

To test the viability of this hypothesis, neutrophils from healthy controls (n = 3) were incubated with opsonized *Escherichia coli* for 1 h. Supernatants were assayed for effects on vascular tone in explants from rat superior mesenteric arteries[481,482]. Prior to all experiments, vessels showed normal contractile responses to the adrenergic agonist phenylephrine (EC_{50} = 0.15 μM); mural tensions returned to baseline after washing. The phenylephrine dose-response was then investigated in vessels pre-incubated for 1 h with the neutrophil supernatants. A reduction in maximum tension but not EC_{50} was observed (Fig. 6.8), indicating non-competitive inhibition of contraction. There was no evidence of vessel damage. Controls were rings from the same vessels not pre-exposed in this manner.

Serum from subjects taken 24 h after injection was also measured for free circulating LPS. None was detected in the majority of samples; this might relate to insufficient assay sensitivity or choosing too late a time point.

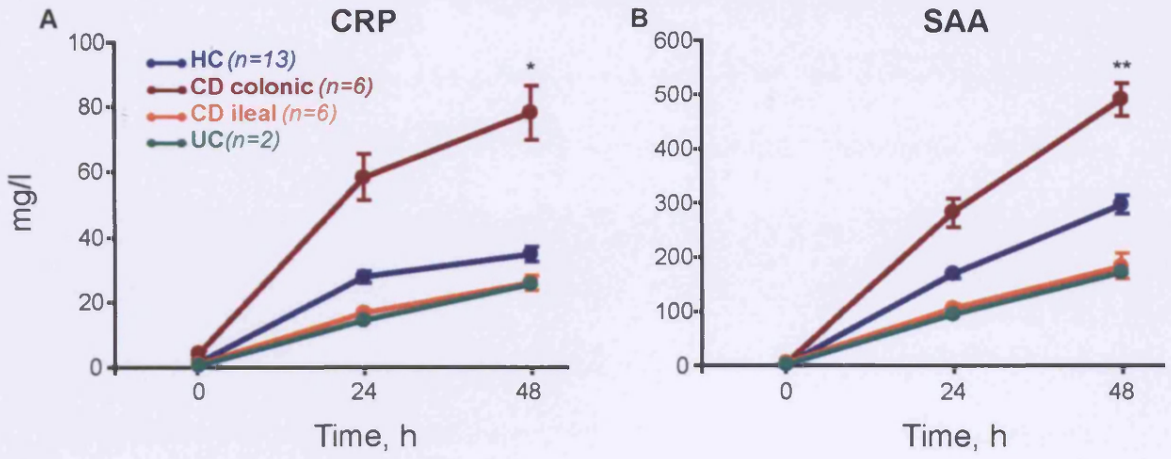


Figure 6.7 Serum acute phase reactants. **(A)** CRP. **(B)** SAA. Results shown as mean ± SEM. Significance values compared to HC at same time point.
HC: healthy controls; CD: Crohn's patients; UC: ulcerative colitis patients
*$P < 0.05$; **$P < 0.01$*

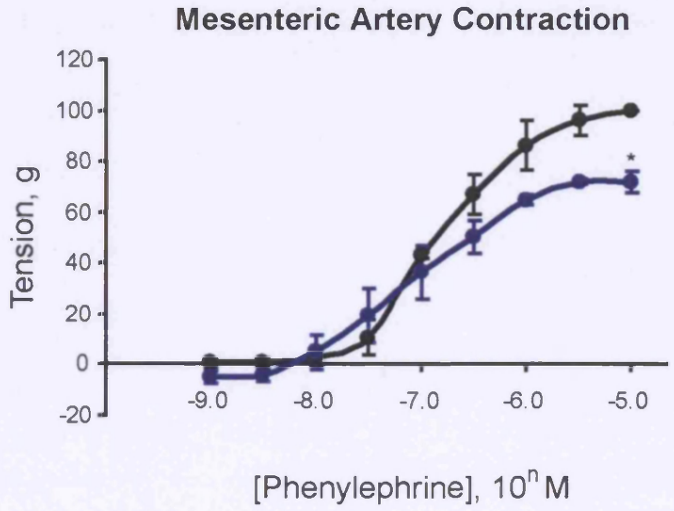


Figure 6.8 Dose-response to phenylephrine in rat superior mesenteric artery. Vessels were pre-incubated with medium alone (black line) or supernatants from healthy neutrophils mixed with opsonized *Escherichia coli* (blue line). Results shown as mean ± SEM. Significance values compared to control at same concentration.
$P < 0.05$

6.2.7 Pharmacological manipulation of blood flow

In animal models, LPS induces vasodilatation in through a nitric oxide (NO)-dependent mechanism[483]. To investigate the relevance of this mediator in the normal elevation of blood flow induced by *Escherichia coli*, 3 healthy controls underwent brachial artery cannulation 24 h following inoculation. Baseline measurements were taken (Fig. 6.9A,E) then noradrenaline infused, with minimal effect (Fig. 6.9B,E). Saline washout normalized blood flow again (Fig. 6.9C,E). The NOS inhibitor NG-monomethyl-L-arginine acetate (l-NMMA) was subsequently infused[382,484], which rapidly attenuated the blood flow response (Fig. 6.9D,E; $P = 0.008$).

The effects of sildenafil citrate (Viagra^{TM}), a cyclic GMP (cGMP) phosphodiesterase-5 (PDE-5) inhibitor and vasodilator[485], were consequently examined. A 50 mg oral dose was administered to 5 healthy subjects and 10 Crohn's patients at 24 h (8 patients) or 48 h (2 patients) after injection. This caused marked elevation in blood flow in most subjects ($P = 0.02$) over the subsequent 60 min (Fig. 6.10A-C,E), particular in those with colonic disease. The effects were transient, returning towards baseline by 90 min (Fig. 6.10D), but supported an NO-related defect. They also demonstrated that this could be overcome using pharmacological treatment (Fig. 6.10E).

6.2.8 Ultrasound analyses

In most participants, discrete masses could be felt at the injection sites on completion of the experiment; their volumes were quantified by ultrasound in a proportion of subjects at 7 days. Masses, where measured, were most substantial in healthy controls (Fig. 6.11A; mean = 2.37 cm^3, standard deviation = 1.25 cm^3,

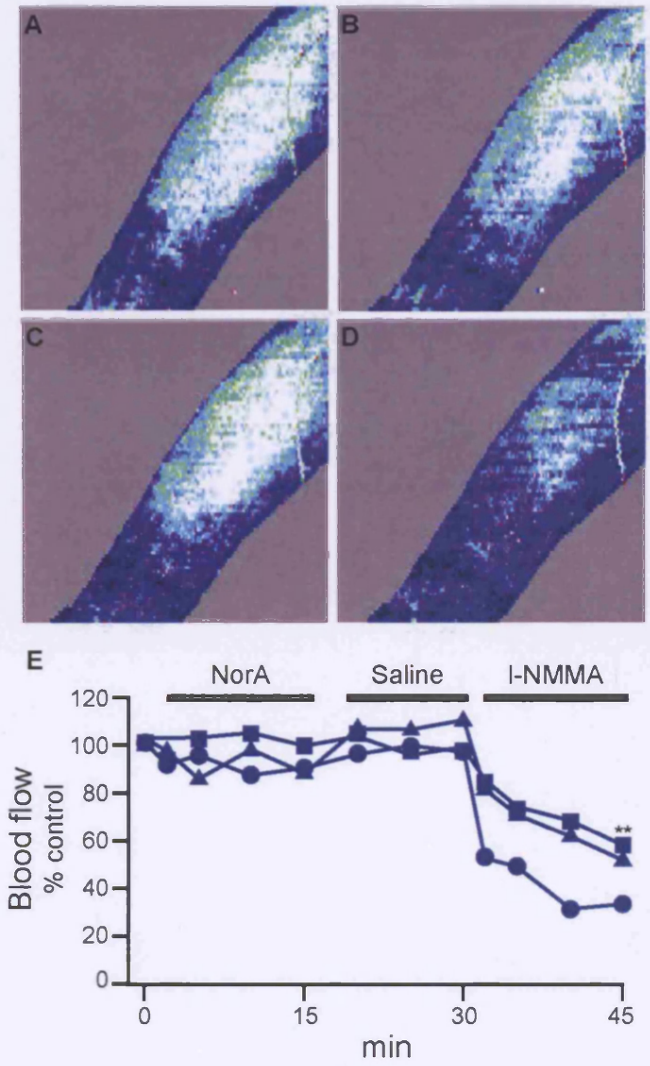


Figure 6.9 Pharmacological manipulation of blood flow in healthy controls. A cannula was inserted into the brachial artery 24 h following bacterial injection, and measurements taken at **(A)** baseline, **(B)** after infusion of noradrenaline (NorA), **(C)** after normal saline washout and **(D)** after infusion of the NOS inhibitor l-NMMA. **(E)** Time course of changes in 3 different healthy controls are shown. Significance values compared to baseline.
*$P < 0.05$

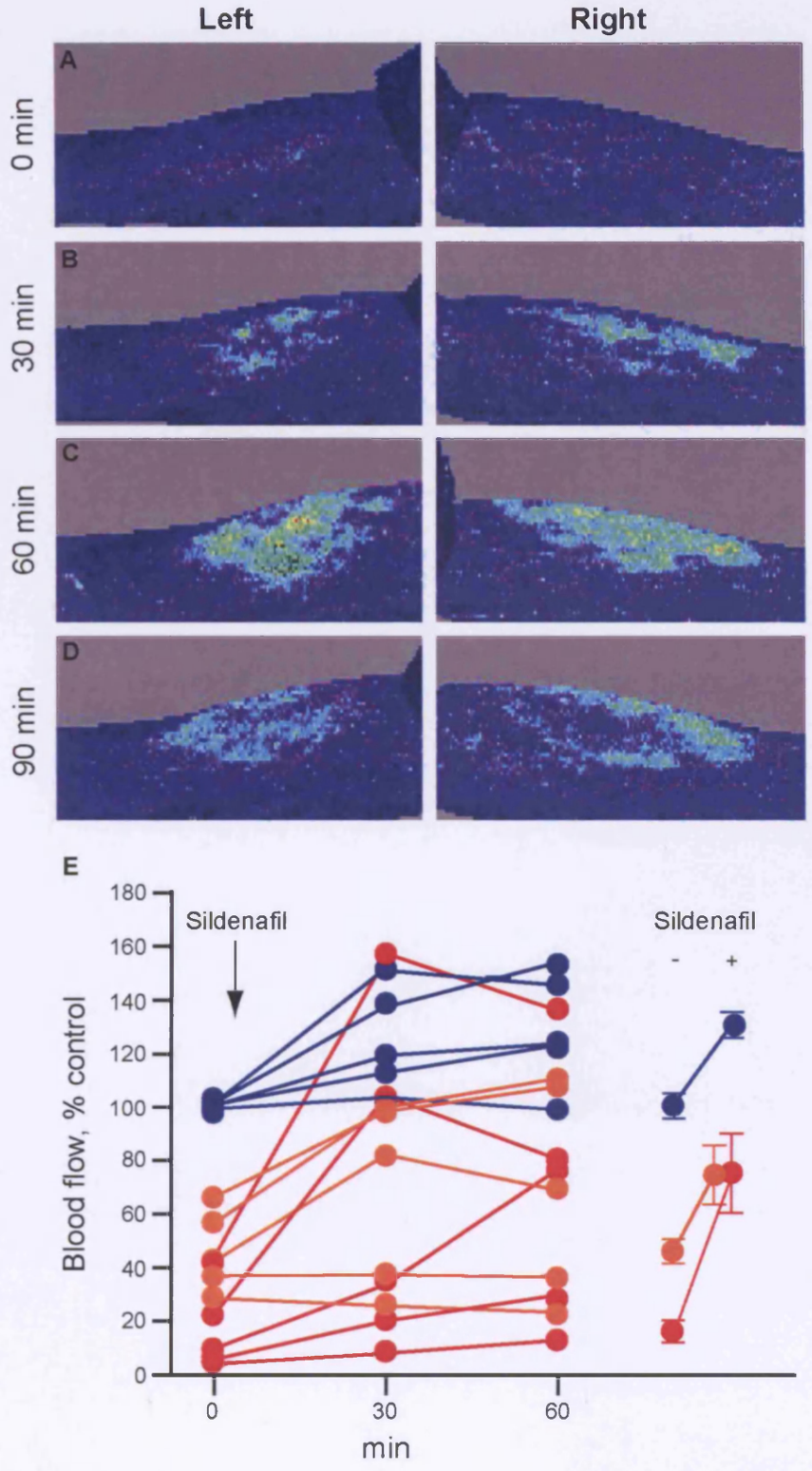


Figure 6.10 Augmentation of blood flow responses by sildenafil citrate. Administration 24 h following bacterial injection. Measurements were taken at **(A)** baseline, **(B)** 30 min, **(C)** 60 min and **(D)** 90 min in a colonic Crohn's patient. **(E)** Time course of changes in healthy controls (blue), ileal Crohn's patients (orange) and colonic Crohn's patients (red). Mean changes ± SEM also shown.

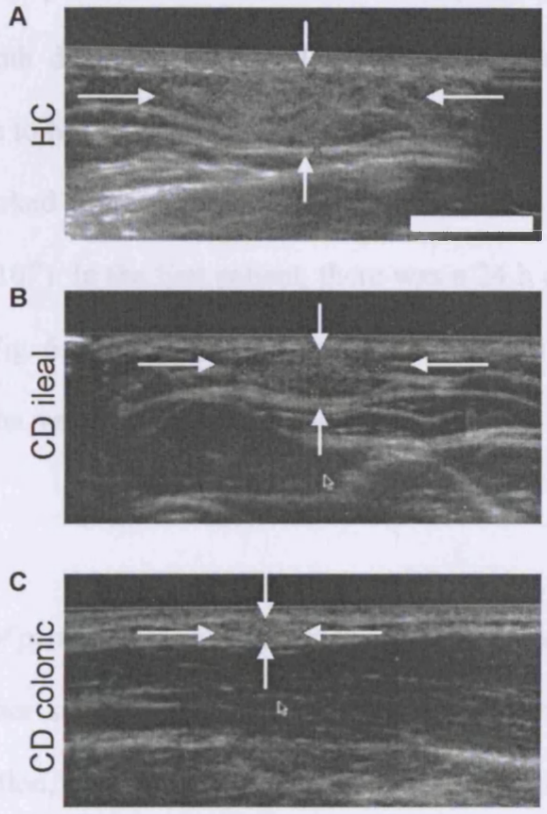


Figure 6.11 Ultrasound visualization of subcutaneous masses 7 days after injection. Representative images from **(A)** a healthy control (HC), **(B)** an ileal Crohn's (CD) patient and **(C)** a colonic CD patient.

n = 5), smaller in ileal Crohn's patients (Fig. 6.11B; mean = 0.65 cm^3, standard deviation = 0.45 cm^3, n = 5), and minimal or absent in colonic Crohn's patients (Fig. 6.12C; n = 5). All masses resolved within 6 weeks.

6.2.9 Delayed resolution of vascular responses in ulcerative colitis

The 2 ulcerative colitis patients enrolled in this study (see Appendix 1: Table A1.10 for details) both demonstrated initial local and systemic inflammatory responses comparable to healthy controls (Fig. 6.2C; Fig. 6.3D; Fig 6.5-Fig. 6.7; Fig. 6.12A-D). In marked contrast, both failed to resolve by 48 h (Fig. 6.12E,F; Fig. 6.3D; $P = 0.6 \times 10^{-6}$). In the first patient, there was a 24 h delay with return to baseline by 72 h (Fig. 6.12G); all other signs of inflammation also remitted. In the second patient, the vascular response was only gradually declining at this time (Fig. 6.12H).

6.2.10 Development of pyoderma gangrenosum in an ulcerative colitis patient

In contrast to all other subjects, in whom inflammation resolved completely within 4 days of injection, it persisted in the second ulcerative colitis patient (Fig. 6.13A). By day 6, it had become increasingly severe, with local vesiculation at each injection site (Fig. 6.13B). This caused clinical concern, and was treated with a single intravenous dose of hydrocortisone and the NSAID diclofenac. Over the subsequent 8 h, vesicles coalesced forming 3 cm diameter painful blisters. These were treated by incision and drainage (Fig. 6.13C), resulting in considerable pain relief after discharge of the non-cloudy serous contents. The latter was assayed for cytokine levels (Table 6.2), of which IL-8 was markedly raised. Systemically, the patient was apyrexial, haemodynamically stable and had

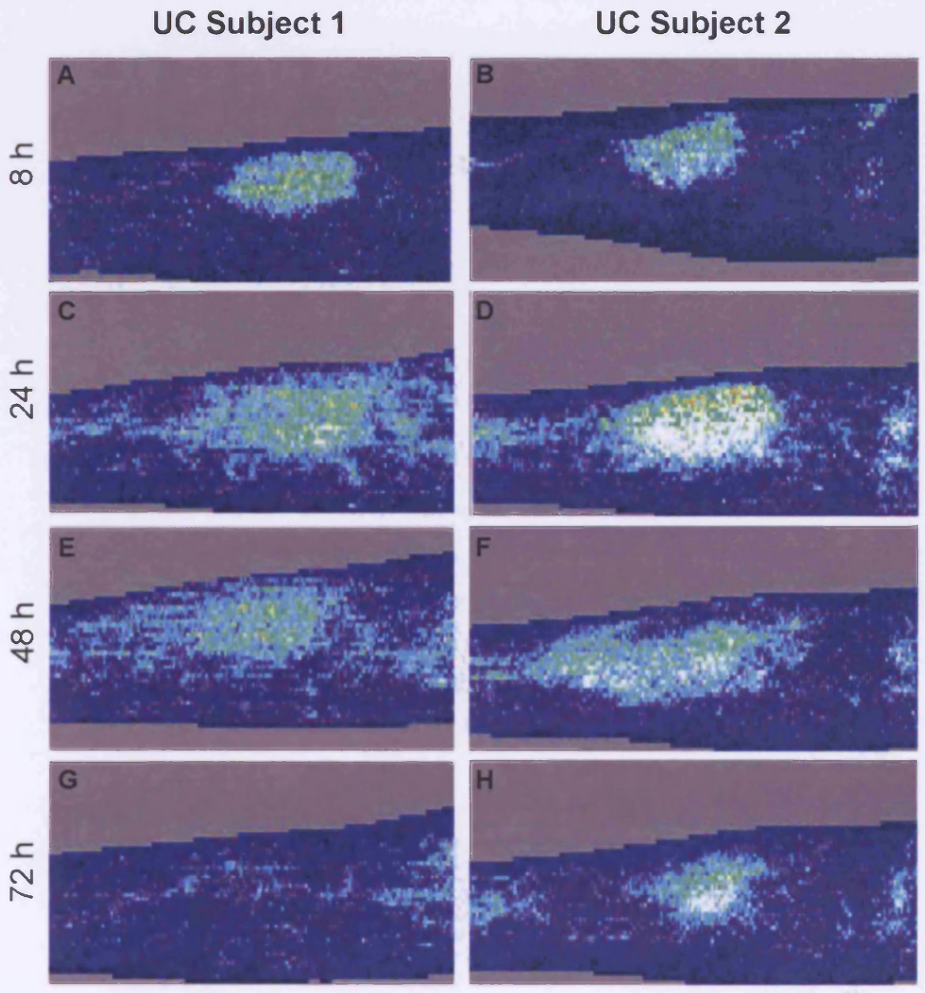


Figure 6.12 Delayed resolution of blood flow response in patients with ulcerative colitis. Measurements were taken at **(A,B)** 8 h, **(C,D)** 24 h, **(E,F)** 48 h and **(G,H)** 72 h following injection of *Escherichia coli*.

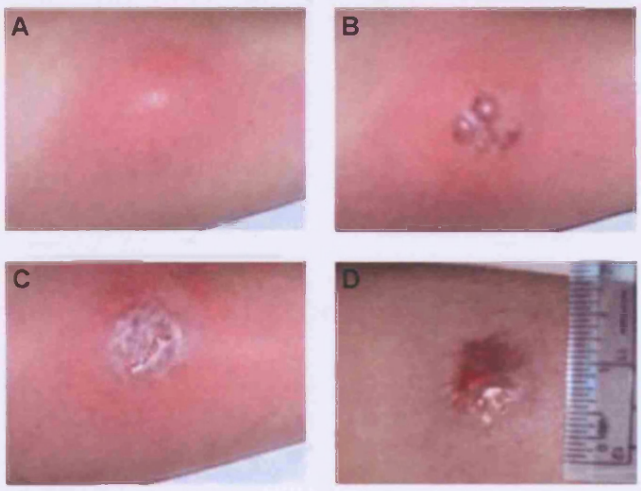


Figure 6.13 Development of vesiculation and ulceration at the site of bacterial injection in a patient with ulcerative colitis. Following a prolonged erythematous reaction (**A**), vesicles developed (**B**) that coalesced to form a single blister. This was incised (**C**), after which the skin became ulcerated with a tender violaceous margin (**D**).

Cytokine	Concentration	Units
IL-1β	3.728	ng/ml
IL-6	1.905	ng/ml
IL-8	1.597	μg/ml
IL-10	37.49	pg/ml
IL-12	Not detectable	
IFN-γ	584.7	pg/ml
TNF-α	404.34	pg/ml
TGF-β	150	pg/ml

Table 6.2 Cytokine concentrations in fluid aspirated from blisters that developed in one ulcerative colitis patient.

no other symptoms (including gastrointestinal). The white cell count was elevated to 10.56 x 10^9/ml (9.38 x 10^9/ml neutrophils) and CRP to 37.3 mg/l. This adverse event was immediately reported to the ethics committee, and studies in ulcerative colitis patients electively terminated.

The following day, two 2.5 x 3.0 cm superficial tender ulcers had developed at both injection sites. These possessed purple margins with surrounding erythema and induration (Fig. 6.13D); a diagnosis of vesicobullous pyoderma gangrenosum was made. A single infusion of 5 mg/kg Infliximab[486] resulted in 50% reduction of the area of each ulcer overnight. Slow healing continued on topical 0.05% clobetasol propionate, although the lesions still appeared markedly inflammatory. Four weeks later, treatment with topical 0.03% tacrolimus[487] induced re-epithelialization and remission of erythema within 2 weeks.

The original batch of injected bacteria was sterile on culture, as were swabs from the ulcers on day 7. At the same time, biopsies were taken from the wound margins, sectioned and stained with haematoxylin and eosin. These showed granulation tissue with active chronic inflammation; marked neutrophil and mononuclear infiltration extending into the subcutis; oedema and fibrin exudation in the superficial dermis; and focal pseudoepitheliomatous hyperplasia (Fig. 6.14A-D). These were consistent with the clinical diagnosis[488].

6.2.11 Potential pro-inflammatory genes in ulcerative colitis

After the prolonged inflammatory reaction developed, the patient recalled a similar episode 6 years previously after operation for an ingrown toenail. The incision site became infected and vesicles arose identical to those on the forearm

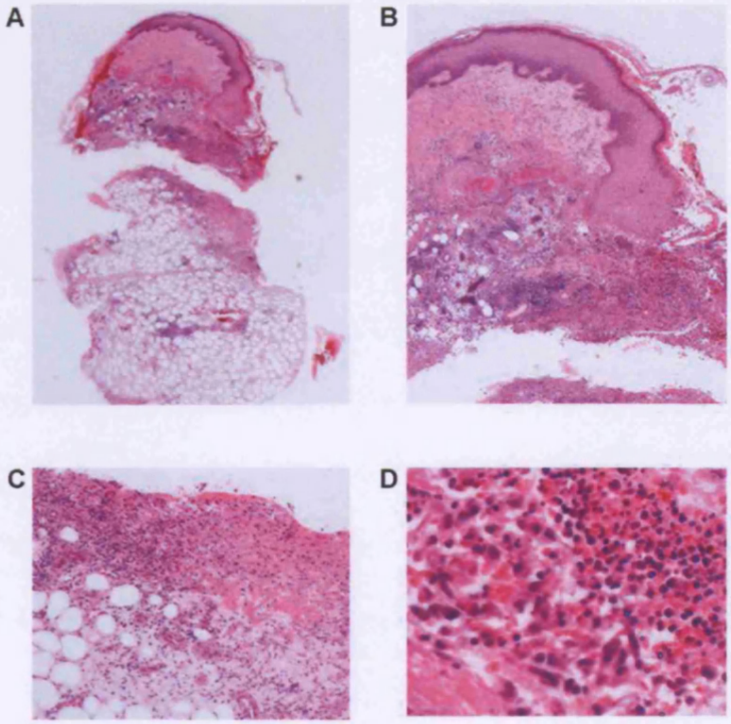


Figure 6.14 Haematoxylin and eosin stained sections from biopsies of the ulcer margin. Samples taken from the skin lesion that developed in one ulcerative colitis patient. **(A)** Low power image showing granulation tissue, inflammation in the subcutis, fibrin exudation into the superficial dermis, and pseudoepitheliomatous hyperplasia. **(B,C)** Medium power views. **(D)** High power image showing extensive neutrophil and mononuclear cell infiltration.

at this presentation. These resolved slowly over two months on steroids and antibiotics, but poor wound healing necessitated debridement. Hospital notes from this prior incident were not available, and the information was not volunteered when originally inquiring about the past medical history. This stereotyped reaction raises the possibility of an underlying molecular abnormality, as opposed to a stochastic reaction. The patient also had a documented Grade 3 reaction on previous Heaf testing, which may be relevant.

Pyoderma gangrenosum can be genetically determined as part of an auto-inflammatory syndrome: pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome[489]. The brother of the patient in this study developed cervical inflammatory arthritis in his third decade, suggesting a potential hereditable syndrome. Consequently, genes known to predispose to auto-inflammatory syndromes (Familial Mediterranean Fever[490]; TNF-Receptor-Associated Periodic Syndrome[491]; Hyperimmunoglobulinemia D and Periodic Fever[492]; Cutaneous Articular Syndrome/Neonatal-Onset Multi-system Inflammatory Disease[493,494]; PAPA syndrome; and Muckle-Wells Syndrome[495]) were sequenced in this patient. No mutations were detected.

6.3 Discussion

6.3.1 Bacterial injection technique

The technique developed here allows investigation of the acute inflammatory response induced by bacteria in humans *in vivo*. Injection of heat-killed *Escherichia coli* was safe in healthy controls and Crohn's patients. Attachment of a radiolabel to the bacteria demonstrated that organisms remain highly localised to the inoculation site prior to clearance. Liberation of this label following

digestion by phagocytes could also be used in future to quantify this clearance, possibly in combination with detection of leukocyte recruitment using ^{111}indium-tagged neutrophils (as employed clinically[496]). Infiltration of high energy indium and disappearance of low energy technetium signals would allow detailed measurements of this acute process. The reproducibility and minimal intra-individual variability of this method are highlighted by the substantial correlations between blood flows in left and right arms at each time point in this study in all subjects.

The side effect profile of the technique, whilst not pleasant, was tolerable. Most resolved by 24 h without medication or on simple analgesia. Subjects were encouraged to take paracetamol for pain relief, and avoid potentially immunosuppressive NSAIDs if possible. The reaction in ulcerative colitis patients was very different from other subjects (see *6.2.10*), resulting in elective termination of the study in this group. Replication of these experiments in such individuals may not be safe under the conditions employed here.

6.3.2 Reduced resistance vessel dilatation in Crohn's disease

In healthy controls, killed bacteria elicited a vigorous inflammatory response. One component of this was a large increase in forearm blood flow, involving dilatation of both resistance vessels[497] (leading to flow augmentation as visualized by laser Doppler) and capillaries[498] (causing erythema). Measurement of flow relied on the Doppler principle, detecting scattering (due to erythrocyte movement) of light from a monochromatic laser directed at the tissue. Photodetection of the frequency broadened light produced a photocurrent, computer processed to provide blood flow measurements proportional to average

erythrocyte velocity. This method samples approximately 1 mm tissue thickness, with measured surface changes representative of the larger tissue volume underneath[377].

A major impairment in blood flow was observed in Crohn's patients, most marked in those with colonic disease and unrelated to *CARD15* genotype. Whereas measured blood flows were grossly different, superficial appearances and areas of erythema were similar in all groups. This indicates an abnormality in relaxation of medium-calibre resistance arteries[498], rather than the capillary bed. The mechanism of this failure could potentially relate to reduced liberation of bacterial products from the cell wall or vasoactive mediator release from leukocytes; recognition of these agents by leukocytes or the vascular endothelium; the response of either of these cells; or an abnormality in the vascular smooth muscle contractile machinery.

Many bacterial cell wall products, including LPS, exert vasodilatatory effects by direct interaction with the endothelium[481,482]. Some of these will be solubilized from bacteria, in part by neutrophils infiltrating the tissues following infection that digest cell walls into smaller muropeptides. Reduced neutrophil recruitment as found in Crohn's disease (see *3.2.4* and *4.2.2*) could diminish release of these mediators. The viability of this hypothesis was demonstrated in the organ bath model, which illustrated that neutrophils normally produce soluble vasoactive products after phagocytosis of bacteria.

6.3.3 Resistance vessel dilatation has an NO-mediated component

In animals, the vascular response to LPS and whole bacteria has been demonstrated to occur through NO release[499,500]. The response to bacteria can be

attenuated by prior neutrophil depletion, through a mechanism reducing cGMP production[500]. NO causes vascular relaxation by diffusing into smooth muscle cells, in which it activates guanylate cyclase increasing cGMP production[501]. The latter reduces intracellular calcium release and leads to dephosphorylation of myosin (through actions on myosin light chain kinase) impeding interaction with actin[502]. These perturbations to the contractile machinery translate into lower vessel wall tensions.

In the human model developed in this study, the response in healthy controls to bacteria could be attenuated by infusing a non-isoform selective NOS inhibitor. Failure of high dose noradrenaline to cause vasoconstriction in the same experiments attests to the specificity of the blockade.

PDE-5 catabolizes cGMP, terminating its action[503]. Consequently, the effects of the PDE-5 inhibitor sildenafil citrate on blood flow were assessed in healthy individuals and Crohn's patients. Most subjects augmented their response following its administration, with a time course consistent with established pharmacokinetics. In a number of Crohn's patients, blood flow was restored to near-normal levels. One possibility is that cGMP production is diminished in these patients, normalized by sildenafil. Another is that a right shift has occurred in its dose-response curve that can be overcome by elevation of cGMP concentrations to supra-normal levels. Direct measurement of cGMP in these studies was not possible, although the experiment could theoretically be performed on vasculature isolated from intestinal resection specimens.

The impaired vascular response was observed in all Crohn's patients, including those off treatment with quiescent disease. The very different response in ulcerative colitis patients argues against a state of tolerance induced by long

term *Escherichia coli* exposure secondary to a chronically inflamed and permeable intestinal wall. The failure of vascular dilatation could therefore represent either a primary pathogenic abnormality or a marker of the underlying process. Previous vascular theories of Crohn's disease have been proposed, suggesting either a vasculitic process[504] or an arteriosclerotic mesenteric ischaemia[505]. Diminished NO production has been reported in Crohn's bowel, but only in regions of active inflammation[506]. These observations are all very different from the abnormality in vasodilatation following exposure to bacteria described here.

Reduced tissue perfusion could contribute to attenuated leukocyte delivery, although normal migration of monocytes and lymphocytes (see *4.2.2*) argues against this exerting a major role. It is more likely to represent a marker of an impaired acute inflammatory response, albeit one that could further compound the underlying abnormality. If this were the case, reversal of this phenomenon by PDE-5 inhibitors might prove therapeutically useful. A trial of the longer-acting agents in this class, such as vardenafil and tadalafil[507], merits investigation.

6.3.4 The systemic inflammatory response in Crohn's disease

Despite the weakened response in the forearm, Crohn's patients mounted a strong systemic acute phase reaction[508]. This confirms that bacteria are recognized and induce some inflammatory sequelae. Interestingly, the systemic response was greatest in colonic Crohn's patients, who exhibited the greatest attenuation of local changes. This illustrates the general principal that failure of local acute inflammation can drive a systemic pro-inflammatory state, with characteristics identical to those observed in active Crohn's disease[509].

Of the serum cytokines measured, only IL-6 was substantial elevated, again to greatest levels in colonic Crohn's patients. IL-6 is principally produced by macrophages, and potently stimulates lymphocyte activation, proliferation and differentiation[510]. It also provides the molecular link between local inflammation and the systemic response, driving production of acute phase proteins by the liver[511]. The rise in IL-6 observed in all subjects 24 h following injection is likely to account for the sustained increases in CRP and SAA, proportional to IL-6 concentrations. These observations are also consistent with findings of high mucosal[512] and serum[513] IL-6 concentrations in active (but not inactive) Crohn's disease, but its normal production by monocytes *in vitro*[509]. This argues that high levels arise secondary to over-stimulation of normal mechanisms, possibly due to persistence of bacteria within the mucosa and consequent increased uptake by mononuclear phagocytes.

6.3.5 Impaired resolution of inflammation in ulcerative colitis

The response to bacteria in ulcerative colitis was very different to that observed in Crohn's patients. The local and systemic reactions over the first 24 h were essentially normal, indicating no abnormality in initiation of inflammation. In contrast, resolution was delayed in both patients studied, by 24 h in the first and considerably longer in the second; the latter developed a serious complication.

Pyoderma gangrenosum is an ulcerating, non-infectious neutrophilic dermatosis of unknown aetiology[514]. It develops in up to 5% of inflammatory bowel disease patients, often coinciding with an exacerbation of intestinal disease. It was once considered pathognomonic of ulcerative colitis[515]. Although the underlying insult usually remains unidentified, a minority of cases may occur

as a pathergic reaction to trauma (typically surgery)[516]. There is one report of ulceration developing at the site of an insect bite[517], but none following exposure to bacteria as found here.

Lesions most commonly develop in 30-50 year olds[518], typically affecting the lower limbs and trunk. It has a characteristic appearance: rapid development and progression of a painful, necrolytic cutaneous ulcer with an irregular violaceous undermined border[488]. A number of clinical variants are recognized, including vesicobullous disease as here, characterized by rapidly evolving painful vesicles and bullae that coalesce with central necrosis and a surrounding halo of erythema. Histopathological findings are non-specific, and include a mixed cellular inflammation with neutrophil predominance; disruption or necrosis of dermal or pannicular blood vessels; peri-vascular deposition of IgM, complement component C3 and fibrin[519,520]; and high levels of IL-8[521], thought to drive the neutrophil recruitment. The low incidence of pyoderma gangrenosum renders randomised controlled trials of treatment impractical. Steroids provide the mainstay of therapy although, as in this case[515], immunosuppressive agents such as Infliximab[486] and tacrolimus[487] (a calcineurin inhibitor) may be preferable. Many other immunosuppressants have some reported efficacy[522-528], and a beneficial effect of heparin has been suggested secondary to reduced neutrophil adhesion and transmigration[517].

In this case, the underlying abnormality is likely to relate to defective termination of the immune reaction to bacteria, or mediators activated specifically in the presence of microbes (such as the alternative or lectin pathways of complement[462], as hinted at in *5.2.8*). The patient in this study had previously undergone multiple venesections, tattooing, acupuncture and ear

piercings, arguing against classical pathergy as seen in Behçet's disease[529]. The stereotyped reaction did, however, previously occur in relation to infection of a surgical wound site, underscoring the importance of a bacterial trigger. Whether this is a general abnormality or specific to coliform organisms remains undetermined.

An impairment in inflammation resolution in ulcerative colitis is therefore implicated, either secondary to defective termination of pro-inflammatory mediator production or activation of counter-regulatory mechanisms such as anti-inflammatory cytokines or resolvins[245] (see *1.5.6*). Either mechanism could account for the sustained elevation in IL-8 concentrations measured in the lesions, which probably drove continued neutrophil accumulation and pathology in this subject. It would also be interesting to determine the presence of T cell subsets in the skin biopsy sections, with particular reference to those with suppressor T_{reg} characteristics.

6.3.6 Defective acute inflammation has functional relevance in Crohn's disease

The results presented in this chapter demonstrate the *in vivo* relevance of the impaired acute inflammatory response in Crohn's patients described in this thesis. They illustrate that a weak local response to intestinal bacteria in the tissues engenders a systemic pro-inflammatory state identical to that observed in active Crohn's disease. A potential novel therapeutic approach using vasodilators such as the PDE-5 inhibitors has been highlighted for evaluation. Furthermore, a hypothesis of ulcerative colitis as a disease of impaired inflammation resolution has been generated, based on preliminary but dramatic pilot data.

Chapter 7: Discussion

7.1 Novel findings in this thesis

7.1.1 Novel findings generated by this research

These studies were undertaken to evaluate the hypothesis that a deficient acute inflammatory response underlies the pathogenesis of Crohn's disease. The work builds on previous data[232] that demonstrated diminished neutrophil accumulation to the sites of skin abrasions in this condition. To this end, a number of novel techniques were developed or evolved, the key findings of which were as follows:

- Serial intestinal biopsies illustrated that attenuated neutrophil recruitment also applies to the bowel, the primary site of disease.
- A modified skin window technique allowed replication of the original data[232] and identified a reduction in cytokine production (particularly IL-8) in Crohn's disease. This was postulated to explain the defect in neutrophil recruitment.
- The novel experiment in which cultured Crohn's macrophages were exposed to wound fluid from healthy individuals revealed an intrinsic defect in their ability to produce IL-8. Characterisation of this fluid suggested that failure of the response to complement (amongst other mediators) might be a central problem in many patients.
- Subcutaneous injection of killed *Escherichia coli* into the forearms of Crohn's patients demonstrated that diminished acute inflammation leads

to functional consequences *in vivo*. These experiments also showed that a weak local response in these individuals could engender a systemic pro-inflammatory state.

7.1.2 Revised schema for the pathogenesis of Crohn's disease

Neutrophils are primarily responsible for the killing and digestion of bacteria, accomplished by the potent digestive enzymes released into the phagocytic vacuole from cytoplasmic granules[205]. They eradicate infecting micro-organisms and are discharged as pus or apoptose[530], resulting in resolution. Macrophages by comparison are also phagocytic but with lower killing and digestive capacity[531]. They play more of a containing role, forming granulomata to wall off foreign material from the remainder of the body, and secrete cytokines that prime and amplify the immune response. This results in florid local and systemic reactions[420].

The hypothesis outlined in Chapter 1 (see *1.5.4*) can now be confirmed and elaborated based on the data presented in this thesis. Although the intestinal mucosa provides a very effective barrier in health, insults such as infection and trauma allow the luminal contents access to the tissues of the bowel wall. In the absence of adequate numbers of neutrophils for effective clearance of bacteria[532], the latter will be taken up by macrophages[474]. This leads to the formation of granulomata[533] and consequently the foci of chronic inflammation containing activated leukocytes (producing TNF-α and other Th1 cytokines) characteristic of Crohn's disease.

The studies performed here indicate that Crohn's disease is associated with failure to translate acute tissue damage into an effective neutrophil response,

substantiating the original hypothesis. The immune system detects bacteria through their interaction with either cell-associated pathogen recognition receptors[534] or soluble serum factors[462]. Precisely how they are sensed within the bowel wall therefore presents a dilemma, since the former are down-regulated in healthy non-inflamed mucosa[42]. One possibility is that bacterial infiltration of the mucosa elicits serum extravasation and subsequent activation of mediators such as members of the complement cascade. The latter induce synthesis of chemokines including IL-8 by resident macrophages (which express high levels of complement receptor[464]), driving recruitment of neutrophils out of the vasculature into the tissues. The diminished response to complement observed here in Crohn's patients impedes this whole chain of events, predisposing to suboptimal acute inflammation and a chronic granulomatous reaction.

7.1.3 The role of CARD15

The discovery that polymorphisms in *CARD15* predispose to Crohn's disease[264,265] generated considerable excitement. These have frequently been afforded a primary role in many pathogenic schemata. To date, only weak mechanistic relationships have been postulated (see *1.6.3*), some contradictory and none proven. Apart from the specific response to MDP, which was normally pro-inflammatory, patients compound heterozygous/homozygous for *CARD15* polymorphisms could not be differentiated from those who were wild type in any experimental model in these studies. This questions the centrality of *CARD15* (see *4.3.4* and *5.3.1* for discussion, including attempted reconciliation of current theories) and suggests that at most it represents one of a number of genetic variations that give rise to a common phenotype.

Instead, the inflammatory response appears to operate on two tiers. The first is a generic reaction following, for example, non-specific damage to the epithelium, achieved through mediators such as the complement cascade. Impairment of this response represented the common defect in Crohn's patients, irrespective of *CARD15* genotype. In the general population, the magnitude and quality of an acute inflammatory reaction will follow a Gaussian distribution. Those at the low tail will be prone to inadequate clearance of luminal constituents infiltrating the bowel mucosa. Fortunately, many of these individuals can augment their response by detection of micro-organisms or their products that ingress through a mucosal defect, using molecules such as CARD15. Under this model, those at risk of developing Crohn's lesions possess attenuation of both the general response and compensatory mechanisms (Fig. 7.1). Polymorphisms in *CARD15* probably predispose to ileal disease[275] as the luminal contents in this region are fluid. In these conditions, a small diffusible product such as MDP (present in high concentrations[404]) would be more likely to penetrate a damaged mucosa than in the large bowel where contents are more solid. It is probable that *CARD15* wild type patients have lesions in other pathogen receptor pathways, a view supported by recent genetic data[323,324,326].

7.1.4 Combination with existing theories of pathogenesis

The assertion that there is an attenuated acute inflammatory response in Crohn's disease would not be mutually exclusive with the majority of data present in favour of alternative hypotheses. The latter broadly divide into those advocating an infectious agent, a primary autoimmunity, or a breakdown in mucosal barrier

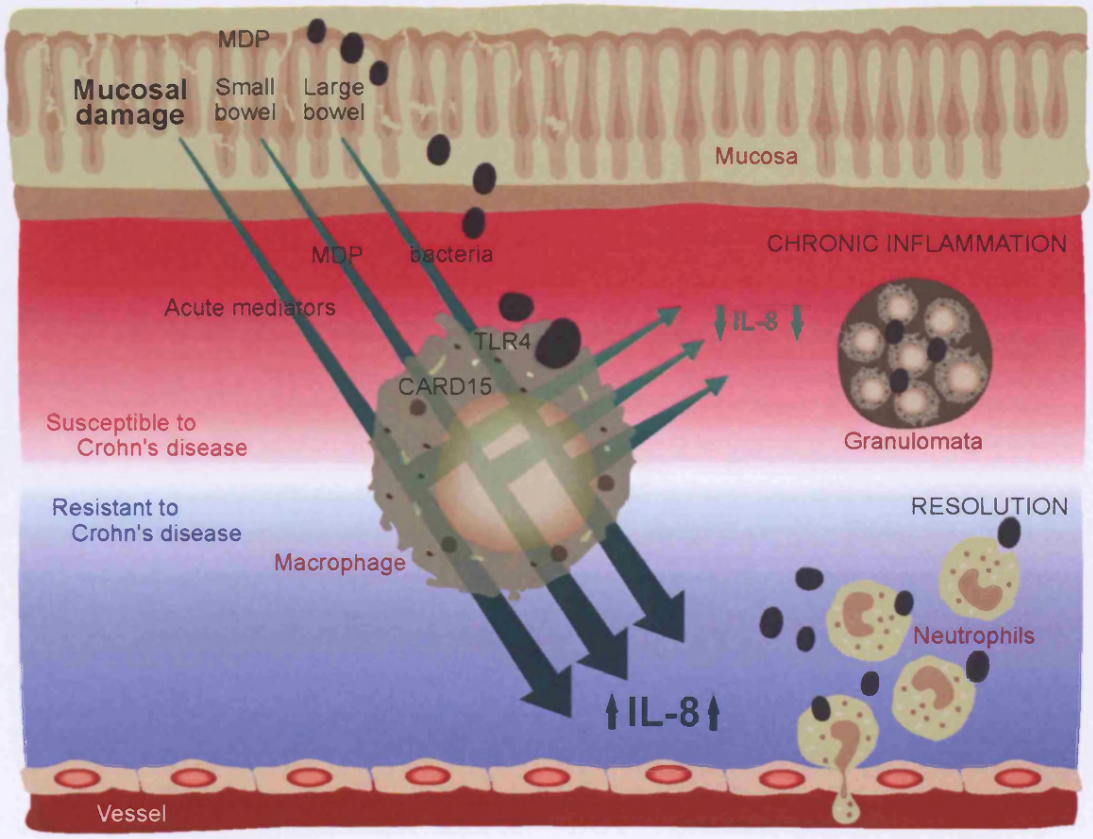


Figure 7.1 Schematic representation of the proposed mechanisms involved in the pathogenesis of Crohn's disease. Damage to the mucosa allows penetration of gut contents including bacteria (brown ovals) into the bowel wall. The outcome depends upon the subsequent inflammatory response. A vigorous response leads to the secretion of high levels of IL-8 attracting large numbers of neutrophils, which phagocytose and digest the bacteria and foreign material. A weak inflammatory response predisposes to Crohn's lesions because the foreign material is taken up by macrophages to form granulomata and foci of chronic inflammation. A naturally weak response can be boosted in the small bowel by MDP signalling through the CARD15 pathway of macrophages to induce them to produce IL-8. In the large bowel a similar role might be performed by TLR4 or CD14. The predisposition to Crohn's disease is greatly increased by a combination of a low innate inflammatory response coupled to failure of one of the compensatory mechanisms.

function (see *1.3*, *1.4.2* and *1.5.1* respectively). Much of the evidence for any of these theories remains indirect and therefore open to interpretation.

Support for infectious aetiologies largely relies on more frequent detection of pathogens in patients that controls. Whereas this could imply increased infection rates, a failure of clearance as postulated here could produce identical results. The apparent lack of pathogen specificity described in Crohn's disease (see *1.3.2*) argues more strongly for the second scenario. The evidence for autoimmunity has been very circumstantial. Findings of autoreactive T cells or broken immunological tolerance[167] could be secondary to longstanding inflammation rather than the primary cause. A disrupted epithelial barrier could also represent a secondary phenomenon, but might additionally compound a weak acute inflammatory response. The adequacy of immune clearance from the bowel will depend not only on leukocyte influx but also on the quantity of foreign material infiltrating the mucosa. Increasing this load could place an already stretched system under excessive strain, beyond its capacity to function efficiently. The familial tendency towards increased bowel permeability described in relatives of Crohn's patients[191] may only manifest clinically once a certain threshold is passed (although evidence for subclinical inflammation has been reported[535]). Overt disease will depend on other parameters determining movement of luminal constituents into the mucosa and potency of the acute inflammatory response.

The hypothesis present here is also consistent with established risk factors. It concords well with the "hygiene hypothesis" (see *1.4.4*), under which the increased incidence of Crohn's disease has been attributed to improved standards of sanitation[177]. The inflammatory response to bacterial ingress would

be much more effective if primed from a state of subclinical inflammation induced by repeated mild infections or parasitic infestation[536] that if started *de novo*. This could also partially explain the preliminary evidence for efficacy of helminthic treatment[180]. Other susceptibility factors include smoking[537] and possibly stress[538]. The former (whilst exerting pleiotropic effects) acts in a generally immunosuppressive manner[539]. It also specifically reduces IL-8 concentrations in the mucosa[540], IL-8 secretion by LPS-stimulated macrophages[541], and compromises mucosal blood flow[542]. All these factors are closely related to rates of neutrophil emigration into the tissues[390,543].

7.1.5 Comparison with CGD

The granulomatous colitis that develops in prototypic neutrophil immunodeficiency conditions such as CGD sets a clear precedent for the existence of such an underlying mechanism. In CGD, the problem relates to impaired bacterial digestion secondary to a defective respiratory burst[212], in contrast to Crohn's disease where inadequate numbers of neutrophils are recruited. The final common effect is nonetheless identical: that the ability of the mucosa to clear foreign material from the tissues becomes overwhelmed, leading to granuloma formation as a protective measure.

The hypothesis that Crohn's disease is secondary to an impaired inflammatory response might suggest that these patients should be abnormally susceptible to infection. Although the condition is not described as such, this does not necessarily weaken the hypothesis. Firstly, the impairment in Crohn's disease is of a partial nature, and neutrophils once recruited can kill bacteria effectively. The abnormality could therefore be limited to digestion and not

stretch to reduced bactericidal capacity. Alternatively, it is possible that Crohn's patients may have heightened susceptibility to infection, but of a subtle nature not yet visible. It may prove necessary to study a large population to detect a positive phenotype, as illustrated in a study of high infectious risk children carrying polymorphisms in mannose-binding lectin[544].

The process of acute inflammation is stochastic in its occurrence with inbuilt redundancy. Even patients with CGD, the most severe of the neutrophil disorders, can live for years or even decades without infection[211]. The difficulty is further compounded because Crohn's patients have varying levels of nutrition, immunosuppressive treatment and surgical intervention. Isolating features integral to the disease from confounding external influences in such a heterogeneous population poses a considerable methodological obstacle.

7.2 Limitations of the study and future directions

7.2.1 Assumptions and general limitations in these studies

A key challenge when investigating chronic conditions such as Crohn's disease remains the distinction of abnormalities representing primary pathogenetic events from those arising as secondary phenomena due to longstanding inflammation or treatment. Although difficult to prove, the fact that the defects reported here occurred in patients with apparently quiescent disease off treatment, in the skin as well as the bowel, and not in inflammatory disease controls argues strongly for the former. Similarly, the fact that macrophages were cultured for 5 days prior to stimulation makes suppression by some humoral or therapeutic factor less likely.

The principal limitation in this study relates to the relatively low numbers of subjects studied in the *in vivo* experiments. Two factors underlie this: the

restrictive selection criteria employed to maximize validity and the nature of the techniques themselves. Most involved a degree of discomfort and all had potential risks and side effects. Given the magnitude of observed differences and high statistical significance levels, expanding numbers was not felt justifiable in most cases. In some instances, explicit number limits were also imposed by the local ethics committee.

An understanding of the molecular mechanisms that give rise to Crohn's disease remains the ultimate goal. Unfortunately, no novel specific genes could be proposed here. The main obstacles lie in the complexity of cellular and signalling pathways involved, and the heterogeneity of Crohn's patients. Data from these studies and others[355,545], based on both clinical and laboratory observations, indicate that it is not a single disease but a syndrome with a variety of predisposing lesions. This would be consistent with a condition characterized by an attenuated acute inflammatory response, orchestrated by multiple factors. A combination of defects in any group of these could elicit the described pathology. An opportunity is nonetheless presented by the studies in this thesis, since a clinical abnormality (failure of neutrophil migration and cytokine production) has been linked to a physiological *in vitro* model (macrophages stimulated with wound fluid). Recapitulation of the latter by exposure to C5a and TNF-α provides specific stimuli in a defined system. This can be used to probe cell signalling pathways thoroughly, in poorly responsive patients identified using an ELISA-based screen.

Finally, it is worth remembering that Crohn's disease is a condition that only affects humans. There is no animal model that reliably reproduces all the features of the condition. Consequently, studies to elucidate the causal

mechanisms have to be conducted almost exclusively on patients and control subjects. Human experimentation is completely different to all other forms of investigation. Subjects must be carefully selected and experiments designed to give answers within the limits of procedures feasible for conduct on human volunteers. In many situations it remains difficult or impossible at a practical level to follow serial changes and obtain a complete description of a phenomenon. For example, to observe the local inflammatory reaction directly following subcutaneous *Escherichia coli* injection would have required serial skin biopsies. Such a procedure would have been neither practicable nor ethically justified in these volunteers. Unavoidably therefore some interpretations have to be inferred following acquisition of as inclusive a data set as possible.

Specific limitations inherent to any particular methodology are discussed in the Results chapters reporting use of that technique.

7.2.2 Therapeutic implications

If accepted, the findings of this thesis may carry important implications for the clinical management of Crohn's disease. At present, immunosuppression provides the mainstay of treatment. Whilst still appropriate for patients presenting acutely in the proposed secondary chronic phase of inflammation, maintenance of remission may prove best accomplished using immune stimulants.

This has already been attempted with GM-CSF[238], which works on a systemic level primarily by promoting granulocyte efflux from bone marrow[546]. Although provisional benefits have been reported, efficacy appeared marginal[238]. Two potential explanations for this were that treatment was administered to

patients with active disease although theoretically lesion prevention would be a more appropriate target, and that GM-CSF does not act locally at the level of the bowel mucosa.

Conversely, IL-8 applied topically corrected the defect in Crohn's patients in the skin window model (see *4.2.5*), as neutrophils can migrate normally when presented with an appropriate stimulus[233]. A similar effect may be achievable in the bowel. IL-8 could be introduced using an oral enteric-coated preparation or by enema. Local synthesis may also prove feasible by genetically modified gut organisms, as has been demonstrated in principle with *Lactococci* producing IL-10[547]. The same could also be achieved by any immune adjuvant that enhances local IL-8 production (see *4.2.6*) or the acute inflammatory response in general. Such mediators are likely to permeate the bowel at regions of mucosal disruption, precisely those areas at greatest risk. Local action should minimize systemic adverse effects. Finally, use of a peptide or protein molecule is probably not contraindicated, as the majority of Crohn's lesions affect regions of the bowel distal to sites of digestion.

The other potential novel therapeutic avenue identified here relates to use of vasodilators, including but not limited to those of the PDE-5 inhibitor class (see *6.2.7*). Although failure of blood vessel relaxation in Crohn's disease may not represent a primary problem (see *6.3.3*), it could compound the underlying abnormality. Given the safety profile of these agents, a pilot clinical trial is warranted, perhaps using the long-acting drugs vardenafil and tadalafil.

7.2.3 Future work

This thesis opens a number of avenues for future research. The key areas are elucidation of the molecular mechanisms underlying the abnormalities described herein and trialling the therapeutic predictions highlighted above (see *7.2.2*). The following would be appropriate for further study:

- Serial biopsies. The numbers of inflammatory disease controls should firstly be increased. Signalling in this model could then be investigated by transcription factor and gene expression profiling of mucosal macrophages and epithelial cells. Ileostomies could be used as an accessible means to perform multiple simultaneous biopsies and determine the effects of immune modulators injected into the mucosa.
- Skin windows. These could be used as a model to test the efficacy of other immune stimulants, as well as to further investigate activation of cell signalling pathways and transcription factors in fractionated exuded leukocytes. The latter studies could be supported by pharmacological agonist and inhibitor experiments.
- Macrophage cultures. Patients could be screened to determine C5a and TNF-α receptor levels. Cell signalling pathways activated by stimulation could be determined by studying protein phosphorylation, either specifically using immunoblots or in general with proteomic methodologies[548]. Transcription factor activation could also be profiled. An attempt should be made to replicate findings in resident intestinal macrophages, as some aspects of their biology and function are known to differ from circulating monocyte-derived macrophages[549]. Any candidate

genes to emerge from these studies could be sequenced. Additionally, given the potential identification of patients whose macrophages may not respond to LPS (see *5.2.1* and *5.2.7*), the endotoxin pathway genes[321,471,534] could be sequenced in these individuals.

- CARD15. Other aspects of macrophage function, for example phagocytosis and respiratory burst, could be assessed for the influence of *CARD15* polymorphisms. Partner proteins might be detectable by attaching recombinant CARD15 to a column subsequently exposed to macrophage cytosol extracts, from cells with and without prior MDP stimulation. Any molecules that bind could by identified by mass spectrometry.
- Bacterial injection. The normal mechanisms of the vascular response could be further characterised by exposing rat superior mesenteric artery sections in an organ bath to *Escherichia coli* with or without neutrophil digestion. The vascular function of mesenteric vessels isolated from Crohn's patient or cancer patient control intestinal resection specimens could also be determined in the same model system. The kinetics of the inflammatory response *in vivo* could be determined using a combination of radiolabelled neutrophils and bacteria (as described in *6.3.1*).
- Other defects in innate immunity. Under this hypothesis, any abnormality that leads to a weak acute inflammatory response could predispose to Crohn's disease. CGD patients appear particularly prone to developing peri-anal disease with fistulation[212]. Crohn's patients with these clinical characteristics may possess a CGD-like abnormality of neutrophil function. Subjects could be screened by looking at a composite measure

such as bacterial digestion, measured by release of a radiolabel incorporated into the cell wall. Any individual showing impairment could then have the process dissected out by assaying phagocytosis (of fluorescently labelled bacteria[550]), respiratory burst (measuring oxygen consumption[551] and cytochrome c reduction[552]), ion fluxes[553] (radioisotope uptake) and digestive enzyme content and function (proteomic methodologies and bactericidal capacity of extracted granules).

- Clinical trials. The therapeutic potential of both IL-8 and PDE-5 inhibitors could be investigated as outlined above (see *7.2.2*). The greatest benefits would be expected in maintenance of remission. Patients who have recently undergone intestinal resections would provide an ideal pilot group, as these individuals normally develop endoscopic recurrence within 1 year.
- Ulcerative colitis. Finally, exciting although preliminary data suggest that the abnormality in ulcerative colitis could relate to a failure of inflammation resolution (see *6.2.9*). These findings should ideally be replicated, although some risk might be involved (see *6.2.10*). It remains unclear whether the adverse event that occurred in one patient was idiosyncratic. Reducing the quantity of bacteria injected might diminished either the probability or magnitude of such a reaction developing again. Should the defect be reproduced, the termination of pro-inflammatory signals in macrophages, neutrophils and lymphocytes (each cell in isolation or co-culture) could be investigated. Analysis of signalling pathways should follow identification of any abnormality. The

hyperphosphorylation of p44/p42 MAP kinase described here (see *5.2.8*) might prove relevant. Counter-regulatory mediators such as the resolvins (see *1.5.6*) may play critical roles, although the molecular techniques to study such systems in detail have yet to be developed.

7.2.4 Conclusion

A hypothesis of Crohn's disease as a form of immunodeficiency has the potential to re-orientate approaches to understand its pathogenesis and the clinical management. Clinically relevant impairments in the acute inflammatory response have been mechanistically linked to defects in cellular function. This research has highlighted a number of areas relevant for future study. These include novel potential therapeutic avenues that might improve maintenance of disease remission. Such an approach could prevent the chronic inflammatory pathology that underlies the considerable morbidity associated with Crohn's disease.

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Appendix 1: Subject Characteristics

A1.1 Demographics

Of the Crohn's patients, 47.8% were male, compared with 54.3% and 58.1% of the healthy and ulcerative colitis controls respectively. The mean age of the Crohn's patients was 44.5 years of age (standard deviation = 15.88, range: 16-87 years old); 43.1 years old in healthy subjects (standard deviation = 18.52, range: 22-84 years old) and 46.4 years in ulcerative colitis patients (standard deviation = 16.59, range: 20-90 years old). The age distributions in each condition are shown (Fig. A1.1A).

The majority of subjects studied in each group were Caucasian, although in Crohn's patients there was a slight over-representation of Jewish subjects with under-representation of people from the Indian subcontinent (Fig. A1.1B). Smoking was more prevalent in Crohn's patients (36.7% current smokers, 11.1 mean pack-year history) compared to healthy controls (18.4% current smokers, 7.3 mean pack-year history) or patients with ulcerative colitis (15.8% current smokers, 6.4 mean pack-year history).

A1.2 Disease characteristics in Crohn's patients

Approximately equal proportions of patients presented with disease involving the ileum, colon or both sites (Fig. A1.2A). The majority had simple inflammatory changes on endoscopy; approximately 20% of the remainder had fibrostenotic disease and a further 20% showed evidence of fistula formation at some stage over the course of their disease, with a small number of individuals displaying

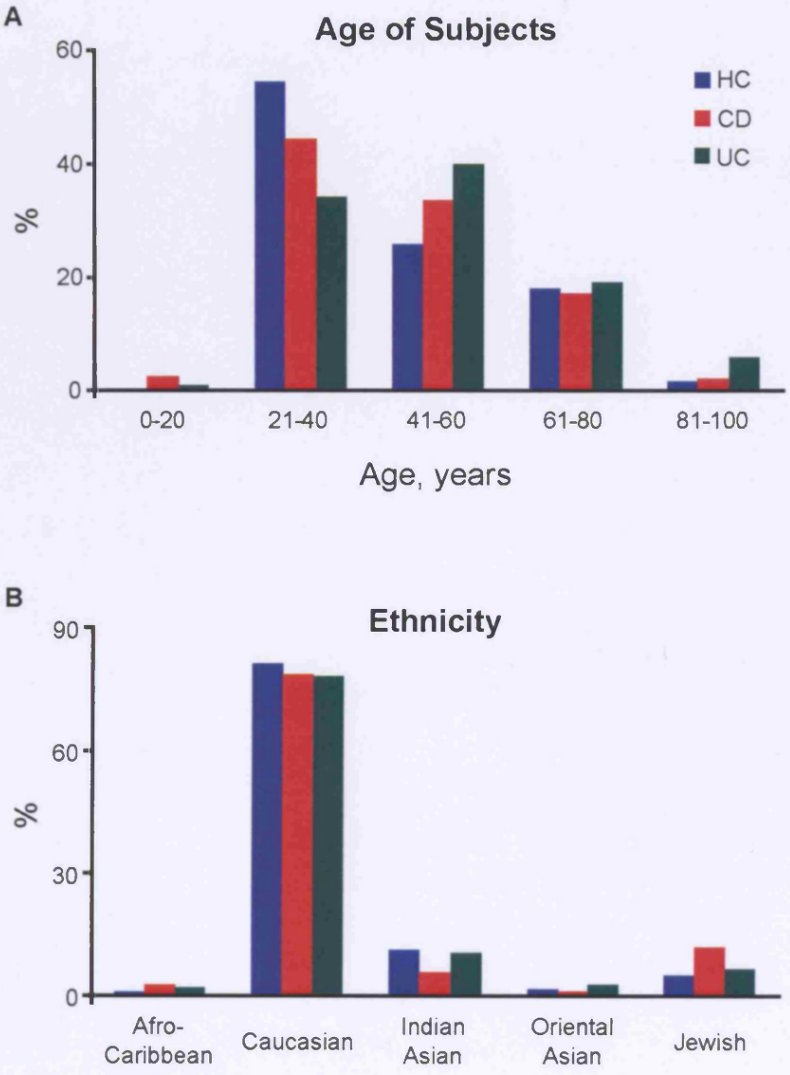

Figure A1.1 Profile of subjects studied. **(A)** Age and **(B)** ethnicity.
HC: healthy controls; CD: Crohn's patients, UC: ulcerative colitis patients.

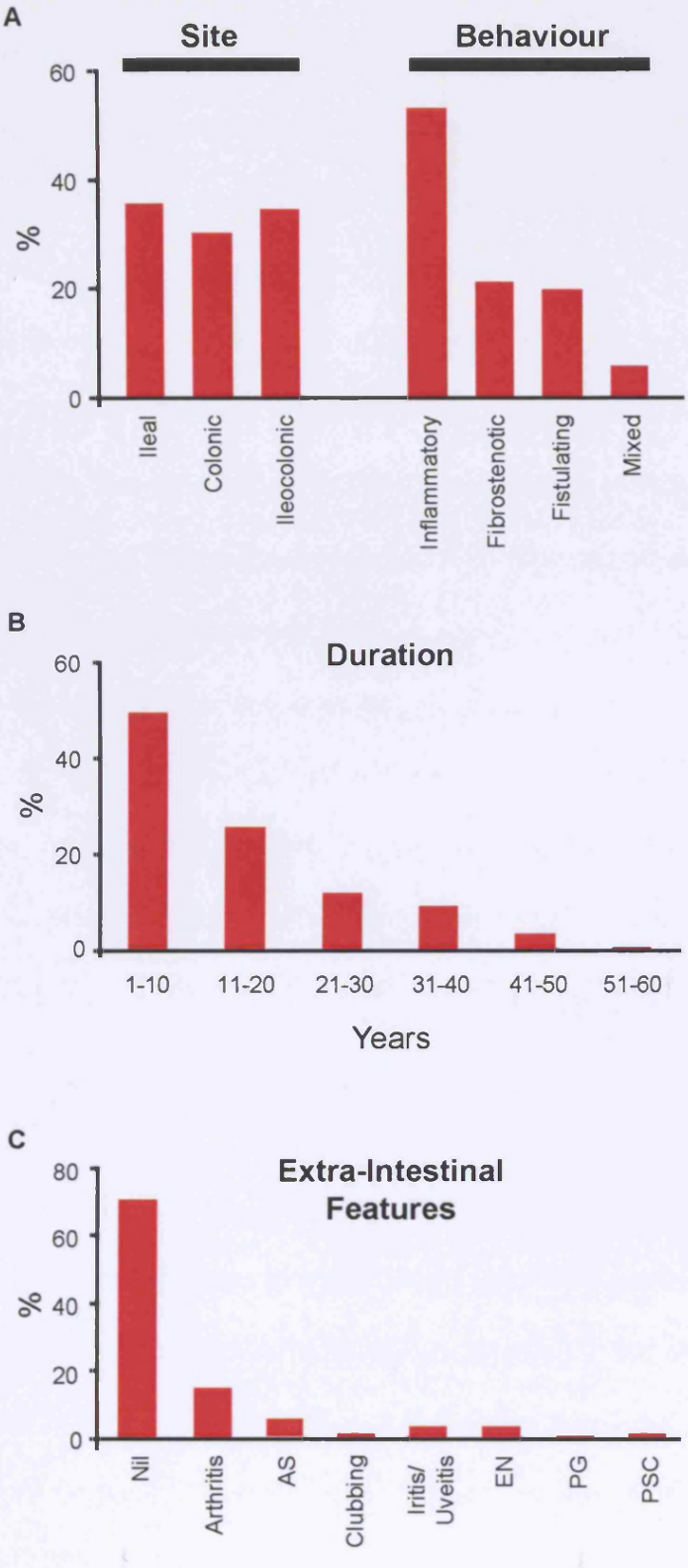

Figure A1.2 Disease characteristics of Crohn's patients studied. **(A)** Site of disease and disease behaviour. **(B)** Duration of disease since first diagnosis. **(C)** Extra-intestinal manifestations.

AS: ankylosing spondylitis; EN: erythema nodosum; PG: pyoderma gangrenosum; PSC: primary sclerosing cholangitis.

both characteristics (Fig. A1.2A). Of the 157 patients for whom histological data was available, 38.2% were reported as showing granulomata on biopsy.

The mean duration of disease in this patient population was 14.5 years (standard deviation = 11.84; range: 1-52 years), with the majority diagnosed within the preceding 20 years (Fig. A1.2B). In terms of family history of inflammatory disease, 17.6% of patients had an affected relative with Crohn's disease, 5.7% with ulcerative colitis, 1.3% with ankylosing spondylitis, and three individual patients had a first degree relative with rheumatoid arthritis, coeliac disease or systemic sclerosis respectively. A significant proportion (43.6%) of patients had required at least one intestinal resection during the course of their lives. A minority (9.0%) developed lesions in the upper gastrointestinal tract, and 23.6% showed evidence of peri-anal lesions. The majority of patients had no extra-intestinal manifestations of disease and, in those who did develop such features, arthropathy was the most frequent complaint (Fig A1.2C).

A1.3 CARD15 genotypes

Good allelic discrimination using fluorescent probes was achieved for each SNP (Fig. A1.3A-C). In the few cases in which results were ambiguous, samples were sequenced directly. Consistent with previous reports[264], the three *CARD15* polymorphisms were overrepresented in the Crohn's population (Fig. A1.4A) with 9.3% of subjects carrying two variant alleles (5.5% compound heterozygous, 3.8% homozygous). This supports the predisposition to Crohn's disease conferred by these genetic variants (χ^2 = 18.77, $P < 0.001$), both by simple heterozygosity (odds ratio = 3.2, $P = 0.03$) and compound heterozygosity or homozygosity (odds ratio = 7.7, $P = 0.03$). A single healthy control was

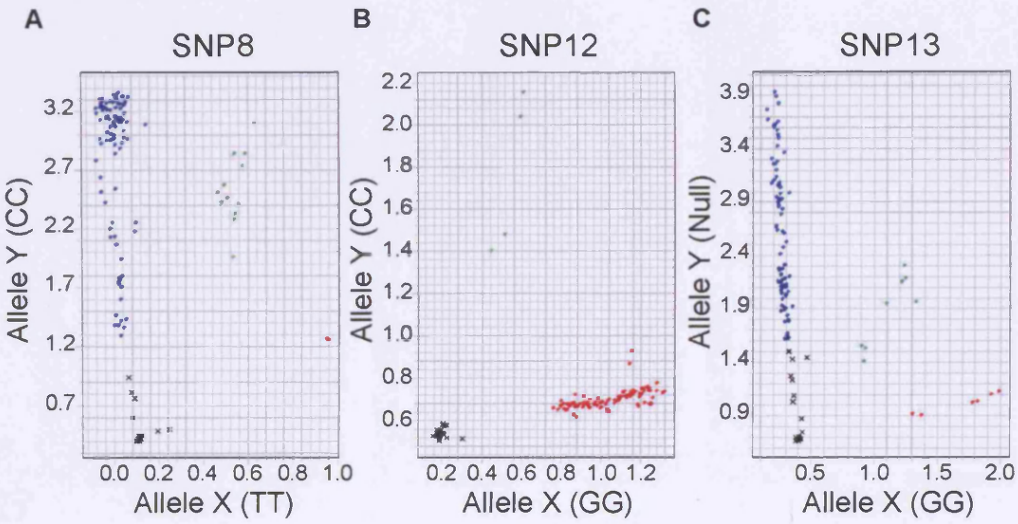

Figure A1.3 Genotyping of subjects by allelic discrimination. **(A)** SNP 8, **(B)** SNP12 and **(C)** SNP13. Axes indicate the degree of fluorescence for each probe. Good discrimination was achieved for each SNP. Each sample was genotyped in duplicate. Those for which a genotype could not be reliably assigned were sequenced directly.
Red points: homozygous for allele X; blue points: homozygous for allele Y; green points: heterozygous crosses: genotype not assigned; squares: negative controls (no DNA).

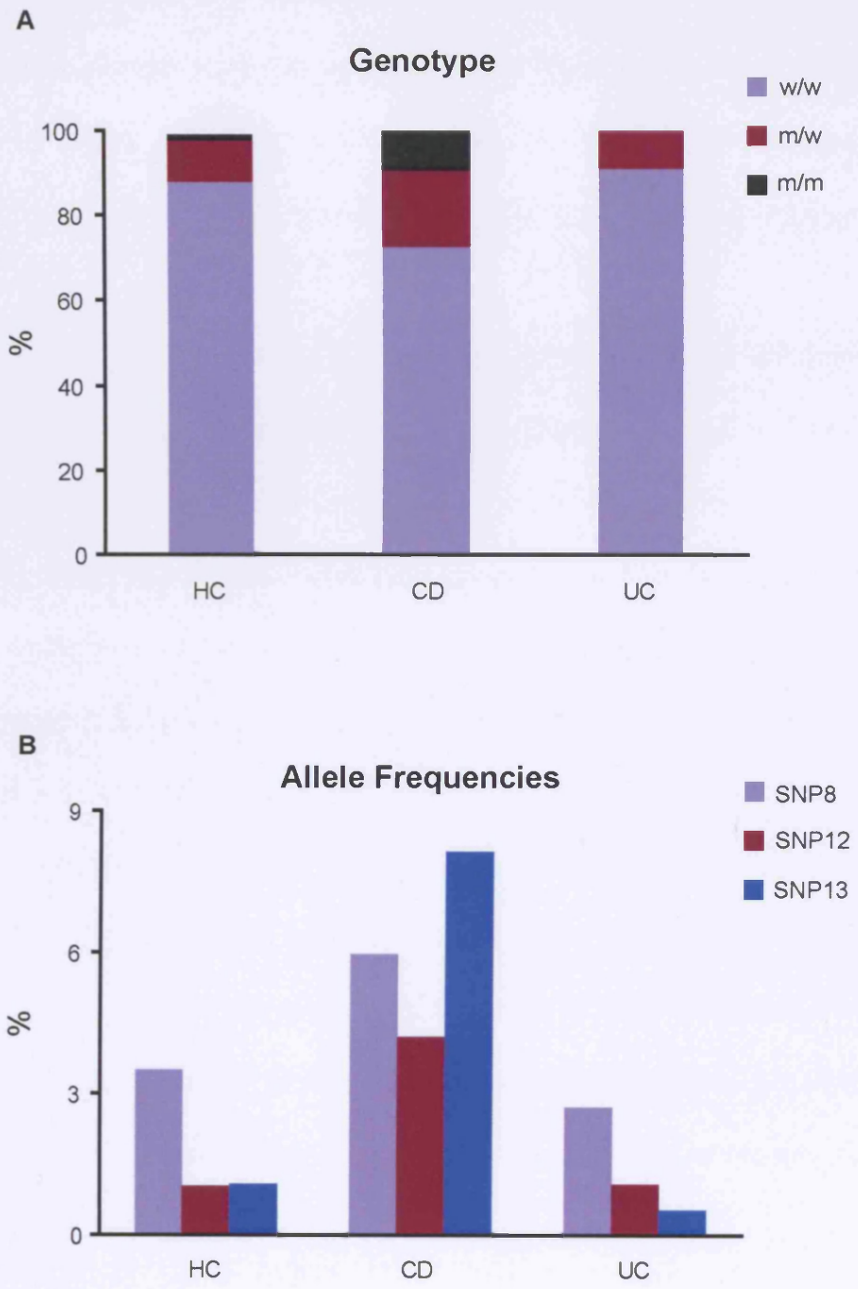

Figure A1.4 *CARD15* polymorphisms. Distribution amongst different subject groups of **(A)** *CARD15* genotype and **(B)** SNPs associated with susceptibility to Crohn's disease.
HC: healthy controls; CD: Crohn's patients, UC: ulcerative colitis patients.

compound heterozygous for SNP8 and SNP13. All three SNPs were more prevalent in Crohn's patients, with most substantial enrichment in SNP13 (Fig. A1.4B). Polymorphisms were almost entirely restricted to Caucasian subjects and people of Jewish extraction: they were not found in any Afrocaribbean or Oriental Asian individuals, and only one Indian patient was simple heterozygous for a single SNP.

Amongst Crohn's patients, there was no difference in the duration of disease based on genotype (Fig. A1.5A). As previously described[275], *CARD15* predisposed specifically to lesions in the ileum (odds ratio = 14.4, $P = 0.008$) (Fig. A1.5B). There was no association with any disease behaviour (Fig. A1.5C); granuloma formation; family history of Crohn's disease; or extraintestinal, peri-anal or upper gastrointestinal manifestations of disease; although patients in this series carrying two polymorphisms had an increased rate of intestinal resection (odds ratio = 3.8, $P = 0.04$).

In addition to the control subjects above, 14 patients with CGD were also genotyped for the *CARD15* polymorphisms. Of these, 10 were wild type, 3 were simple heterozygous, and one patient was compound heterozygous for SNP12 and SNP13. Of these individuals, 5 had intestinal inflammation complicating their CGD of which 4 carried the *CARD15* polymorphisms.

A1.4 Patients studied are representative of the general Crohn's population

A large population of Crohn's patients and controls were genotyped for polymorphisms in *CARD15* known to predispose to Crohn's disease. The groups were largely matched for age and sex. In Crohn's patients, Jewish background

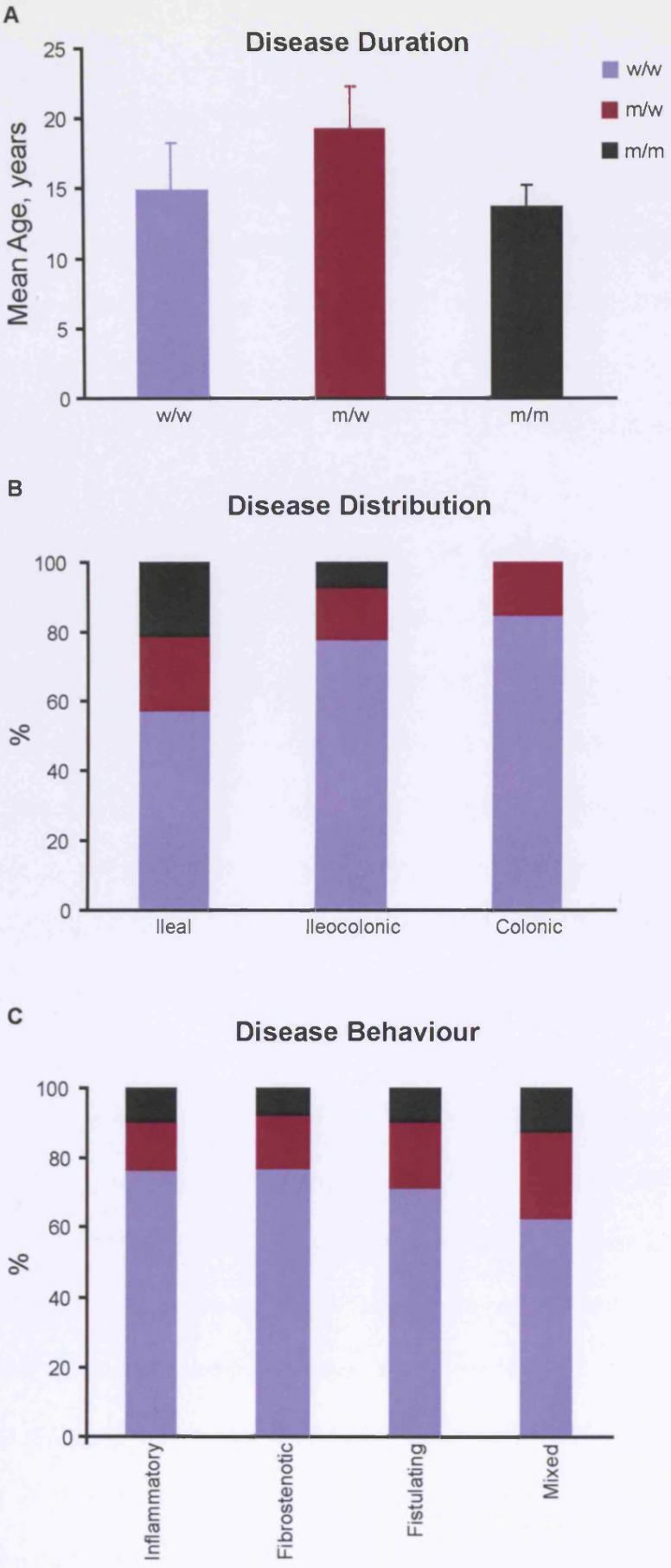

Figure A1.5 Genotype-phenotype correlations. Distribution of CARD15 genotypes in Crohn's patients according to **(A)** time since diagnosis, **(B)** site of intestinal involvement and **(C)** disease behaviour.

was more prevalent, and they were more likely to be current smokers with a greater pack-year history, consistent with established disease risk factors[537,554].

Amongst Crohn's patients, the distribution of lesion sites throughout the gastrointestinal tract was similar to other reported populations[275], as were the clinical disease behaviours[8]; frequency of granulomata in clinical biopsies[13]; and development of overt upper gastrointestinal[267], peri-anal[555] and extra-intestinal manifestations[556].

In concordance with previous studies, the prevalence of *CARD15* polymorphisms was greater in Crohn's patients than in non-inflammatory bowel disease controls or patients with ulcerative colitis[264,265,557], supporting the hypothesis that these predispose to Crohn's disease. We found lower SNP frequencies than those quoted in earlier reports[264], although subsequent studies have documented substantial variation in their occurrence between different Caucasian populations[268-272], and their absence in people of Oriental[273,274] or Afrocaribbean background[558,559]. The presence of a significant number of individuals of these ethnicities in this study could have further diluted the allele frequencies: polymorphisms were almost exclusively carried by Caucasian or Jewish subjects. In addition, we detected one healthy individual carrying two polymorphisms in *CARD15*. This was a male in his early 20s, just before the peak age of onset for Crohn's disease. It is therefore impossible to state whether this individual is a true healthy control or someone who may develop inflammatory bowel disease in later life. A number of individuals in their ninth decade have been reported carrying two polymorphisms with no history of gastrointestinal problems over their lifetime[266], illustrating that *CARD15* variants are not sufficient to induce bowel inflammation.

In patients in this study, carriage of *CARD15* variants predisposed to disease involving the ileum. Unlike some[276,560,561] but not all[268,562] previous reports, there was no association with any other disease characteristic, in particular fibrostenotic behaviour, although patients carrying two polymorphisms may be more prone to requiring intestinal resection.

Finally, an interesting observation was made on genotyping subjects with CGD. In this genetic disorder of neutrophil function, approximately 30% of patients develop inflammatory bowel disease almost indistinguishable from Crohn's disease[216]. In this very limited sample, the number of heterozygotes was unexpectedly high (28.57%) compared to 9.78% and 8.89% in healthy controls and ulcerative colitis patients. It is not known why only a proportion of CGD patients develop colitis. The apparent association with *CARD15* polymorphisms is therefore of interest and may illustrate a requirement for at least two molecular lesions. Of the wild type CGD patients, only one developed colitis. Whilst this might be a true negative, there are a number of rare mutations in *CARD15* not genotyped in this study but which predispose to Crohn's disease[264], and it would be worth sequencing the gene in this subject.

A1.5 Clinical characteristics of subjects for in vivo studies

The clinical characteristics of subjects for the serial biopsy (Tables A1.1-A1.3), skin window (Tables A1.4-A1.7) and bacterial injection studies (Tables A1.8-A1.10) are provided.

Non-Inflammatory Bowel Disease Controls														
ID	Diagnosis	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Post-operative rectal pain	57	F	N	0	Hormone replacement therapy							w/w
2	Gastric carcinoma	63	M	N	80	Metoclopramide							w/w
3	Irritable bowel syndrome	51	F	N	0	Mebeverine							w/w
4	Angiodysplasia	61	M	N	40	Omeprazole							w/w
5	Asymptomatic, family history of colorectal cancer	63	F	N	20	Salbutamol Methyldopa Senna							w/w
6	Irritable bowel syndrome	48	F	N	0	Nil							w/w
7	Asymptomatic, family history of colorectal cancer	48	F	N	0	Nil							m/w
8	Haemorrhoids	52	F	N	0	Nil							w/w
9	Familial adenomatous polyposis	59	M	Y	2	Penicillin V							w/w
10	Familial adenomatous polyposis	56	M	N	5	Simvastatin							w/w

Table A1.1 Characteristics of non-inflammatory bowel disease controls in serial biopsy study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Crohn's Patients													
ID	Site & Behaviour	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Ileal	66	M	N	20	Nil	4.9	<0.5	7	44	573		m/m
2	Ileal, stenotic	37	F	N	10	Nil							m/m
3	Ileocolonic, stenotic	49	M	Y	20	Metronidazole	11.8	3.1	3	43	477	273	m/m
4	Ileal	28	F	N	0	Nil		2.8		47	253		m/m
5	Ileocaecal	54	F	N	0	Nil	5.2	<0.5	8	46			w/w
6	Ileocaecal	51	F	N	20	Nil	4.8	<0.5	8	43			w/w
7	Ileocolonic, previous fistulation	36	F	Y	21	Oral contraceptive pill	13.3	<4.0	2	46		206	w/w
8	Ileocolonic, previous fistulation and pan-proctocolectomy	44	F	Y	3	Nil	7.3	<4.0	10	43		165	w/w
9	Ileocolonic, previous fistulation	52	F	N	0.5	Mesalazine	8.4	<4.0	30	42		533	w/w

Table A1.2 Characteristics of Crohn's patients in serial biopsy study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Ulcerative Colitis Patients													
ID	Extent	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Left-sided	27	M	N	0	Sulfasalazine							w/w
2	Proctitis	72	M	N	0	Mesalazine Enalopril Allopurinol Aspirin Simvastatin	11.2	23.0	5	48			w/w
3	Left-sided	62	F	N	0	Mesalazine	3.52	<5.0		46			w/w

Table A1.3 Characteristics of ulcerative colitis patients in serial biopsy study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Non-Inflammatory Disease Controls													
ID	Diagnosis	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Healthy	23	M	N	0	Nil							m/w
2	Healthy	61	M	N	0	Nil							w/w
3	Healthy	55	M	N	0	Nil							w/w
4	Healthy	39	M	Y	20	Nil							w/w
5	Healthy	49	M	N	0	Nil							w/w
6	Healthy	23	F	N	0	Nil							w/w

Table A1.4 Characteristics of healthy controls in skin window study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Crohn's Patients													
ID	Site & Behaviour	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Ileocaecal, stenotic	39	F	N	10	Nil							m/m
2	Ileocaecal	28	F	N	0	Nil	5.20	<0.5	8	46			m/m
3	Ileocaecal	47	F	N	4	Nil	5.00	<0.5	13	47	239	333	m/m
4	Ileocaecal	48	M	N	40	Nil	5.50	<5.0	8	50	422		w/w
5	Colonic	26	M	N	0	Nil	7.10	2.4	9	49			w/w
6	Ileocaecal	53	M	N	0	Nil	6.50	4.6	5	52	187	252	w/w
7	Colonic	69	M	N	0	Nil	8.60	2.8		45	289	486	w/w
8	Ileocaecal	37	M	N	0	Nil							w/w
9	Ileocolonic, previous fistulation	53	M	N	0	Nil	5.60	1.5	8	49	519	542	m/m
10	Ileocaecal	18	M	N	0	Nil	4.90	<5.0	7	45			w/w
11	Colonic	47	M	N	5	Nil	4.36	6.0		46			w/w
12	Ileocolonic, previous fistulation	70	F	Y	30	Aspirin Loperamide Buscopan	5.75	<5.0	13	44	626		w/w
13	Ileocaecal, stenotic	43	M	Y	20	Nil							w/w

Table A1.5 Characteristics of Crohn's patients in skin window study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Ulcerative Colitis Patients													
ID	Extent	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Pan-colitis	38	M	Y	15	Nil							w/w
2	Pan-colitis	54	M	N	5	Mesalazine							w/w
3	Proctitis	42	F	N	0	Nil							w/w

Table A1.6 Characteristics of ulcerative colitis patients in skin window study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Rheumatoid Arthritis Patients													
ID	Diagnosis	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Rheumatoid arthritis, sicca syndrome	38	M	N	7	Nil							m/w
2	Rheumatoid arthritis	48	F	N	5	Nil							w/w
3	Rheumatoid arthritis	42	F	N	3	Nil							w/w

Table A1.7 Characteristics of rheumatoid arthritis patients in skin window study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Non-Inflammatory Disease Controls													
ID	Diagnosis	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Healthy	24	F	N	0	Nil	8.98	0.4					w/w
2	Healthy	26	M	Y	2	Nil	6.25						w/w
3	Healthy	41	M	N	0	Nil	5.37	3.4					w/w
4	Healthy	24	M	N	0	Nil	6.29	1.3					w/w
5	Healthy	61	M	N	0	Nil	5.16	1.0					w/w
6	Healthy	27	F	Y	2	Nil	6.91	0.3					w/w
7	Healthy	28	F	Y	3	Nil	7.07	1.0					w/w
8	Healthy	43	M	Y	10	Nil	4.26	0.5					w/w
9	Healthy	45	M	N	0	Nil	4.40	0.7					w/w
10	Healthy	22	F	N	0	Nil	5.87	1.1					w/w
11	Healthy	21	F	N	0	Nil	6.26	1.3					w/w
12	Healthy	25	M	Y	0.5	Nil	6.50	1.8					w/w
13	Healthy	21	M	N	0	Nil	4.41	0.4					w/w

Table A1.8 Characteristics of non-inflammatory bowel disease controls in bacterial injection study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Crohn's Patients													
ID	Site & Behaviour	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Ileocaecal, stenotic	39	F	N	10	Nil	9.30	<5.0		49	398		m/m
2	Colonic	41	F	N	5	Nil	6.46	<5.0	5	47	349		w/w
3	Colonic	26	M	N	0	Nil	6.39	<5.0		38	400		w/w
4	Ileocaecal	67	M	N	0	Mesalazine Ibuprofen Codeine Paracetamol	6.18	<5.0	5	47	728	542	m/m
5	Ileocaecal	43	F	N	0	Azathioprine Pentasa	5.87	<5.0	8	46	229		m/m
6	Ileocaecal, stenotic	26	F	N	0	Mesalazine	5.69	<5.0	7	46	374		w/w
7	Ileocolonic, previous fistulation	71	F	Y	30	Aspirin Loperamide Buscopan	7.25	1.8	15	45	375		w/w
8	Ileocolonic	54	F	Y	70	Nil	6.91	4.5		43	482		m/w
9	Colonic	44	M	Y	10	Nil	7.79	2.4	5	42	508		w/w
10	Colonic	43	M	N	0	Methotrexate Mesalazine Metronidazole	8.77	1.4					w/w
11	Colonic	69	M	N	0	Mesalazine	8.98	1.5		45	289		w/w
12	Colonic	65	M	Y	38	Mesalazine Lansoprazole	9.31	3.7	5	42	532		w/w

Table A1.9 Characteristics of Crohn's patients in bacterial injection study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Ulcerative Colitis Patients													
ID	Extent	Age	Sex	Current Smoker	Pack-Year History	Medication	WCC ($x10^9$/L)	CRP (mg/L)	ESR (mm/h)	Albumin (g/L)	B12 (ng/L)	Red Cell Folate (μg/L)	*CARD15* Genotype
1	Pan-colitis	50	F	N	15	Polyethylene glycol '3350'	6.58	0.6	10	49			w/w
2	Left-sided	23	F	N	0	Olsalazine	4.84	0.7	7	48			w/w

Table A1.10 Characteristics of ulcerative colitis patients in bacterial injection study.
WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; w/w: wild type; m/w: simple heterozygote; m/m: compound heterozygote/homozygote.

Appendix 2: Microarray Data

A2: Raw and normalized data

The following tables present data from the microarray experiments in Chapter 5, first before normalization then scaled values. Genes and ESTs for which at least 2-fold differences in expression were detected are provided, regardless of statistical significance. The data set covers each group of subjects in the relevant pools, with and without MDP; labels refer to Affymetrix annotations. Methods for data collection and normalization are detailed in Chapter 2 (see *2.5.4* and *2.5.5*).

Table A2.1: Pre-Normalisation Data

	NIL			MDP				NIL			MDP			NIL	MDP	NIL	MDP	
Label	HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD4	CD5	CD6	CD7	CD8	CD9	Colonic	Colonic	Description
210390_s_at	4.3	7.7	8.4	204.2	207.8	180.3	2	16.7	4.1	29.5	18.4	0.9	1.5	168.4	11	107.4		MIP-1delta
206025_s_at	28.8	52	17.3	1181.8	849.5	1640.5	66.2	25.8	73.3	110.1	48.9	72	61.3	2037.9	47.5	1608.8		TSG-6
206026_s_at	65.6	96.7	17.7	1228.3	1017.4	1664.3	128.8	67.2	163.1	212	72.4	143.6	111.9	1759.3	96.9	1523		TSG-6
202859_x_at	22.6	135.4	44.3	1925.7	2448.7	2706.7	93.9	200.3	126.9	388.6	345.3	152.9	156.3	2299.2	278	2539.6		IL-8
205067_at	45.9	151.9	111.9	2246.2	3314.5	3723.4	175	90.9	90.5	324.7	484.3	76.1	120.3	2954	142.9	2727.9		IL-1beta
39402_at	42.4	142.3	72.3	1494.1	2092.7	2962.2	136.7	97.4	46.8	156.9	267.2	80.5	86.9	2267	136.3	2122.6		IL-1beta
203708_at	14.6	101.2	32.9	319.2	387.3	415.7	51.8	70.6	27	68.2	41.9	29.8	57.9	636.5	39	285.5		PDE-5
219424_at	2.1	21.5	1.8	140.3	281.1	113.5	3.4	2.3	1.1	24.9	15.5	1.8	3.7	62.1	27.5	54.6		EBI-3
221541_at	25	52.3	31.2	177.4	267.5	488	25.2	30.9	30.3	70.8	54	34.3	39.8	409.7	30.2	190.6		Hypothetical Protein
211506_s_at	15	29.1	13.8	526.2	1735.8	2032.6	14.8	30.2	38.9	65.8	73	35.1	34.3	1651.8	66.3	1457.9		IL-8
210029_at	99.6	925.3	16.8	2364.7	1957.9	2750.6	69.7	52.2	15.1	490.4	86.8	15.3	109.9	2101.5	37.3	333.9		IDO
211302_s_at	16.9	77.3	30.6	123.3	184.9	258.3	36.3	32.9	16.9	38	42.5	17.5	31.5	324.3	21.1	122.8		PDE-5
204224_s_at	138	625.5	95.8	1100.9	999.2	1165.8	111.7	132.8	86.2	250.1	74.8	91.4	222.9	807.8	88.7	580.8		GTP Cyclohydrolase
205569_at	14.5	77.1	2.2	195	364.2	169.5	32.1	32.1	24.2	23.9	19.6	30.1	23.5	73.1	42.6	55.9		TSC403 protein
205476_at	4.1	20.1	3.8	669.9	141.3	1232.2	2.3	17.6	2.3	30.4	8.1	4.8	4.5	1629.9	4.7	898.4		MIP-3alpha
210118_s_at	10	21.6	23.6	75	365.7	460.7	25.2	11.4	14.8	4.5	35.6	8.3	13.8	566.8	18.6	127.2		IL-1alpha
205692_s_at	73	313.3	33.4	455.3	563.2	927.2	21.7	64.4	19	106.3	56.9	25.5	92.8	512.8	23.8	190.2		CD38
207850_at	1.3	6	8.5	56.5	171.9	564.2	12.3	8.3	1.6	8.9	15.7	4.2	5.7	697.3	10.3	300.7		GRO-3
205220_at	40.2	112.4	17.9	602.1	117	507.5	21.9	22.7	21.1	152.6	42.9	10.7	67.4	245.4	22.4	60.4		HM74
202357_s_at	35.9	89.4	6.6	104.9	140.1	240.5	14.3	33.2	23.6	58.7	15.9	17.7	36.6	140.1	32.2	74.8		Properdin
204748_at	16.2	12.8	12.7	327.2	41.8	515	20.6	12.5	7	8.5	21.2	19.9	14.5	983.4	12.6	570.2		COX-2
214974_x_at	2.5	15.2	3.2	46.4	535.9	1390.1	111.8	8.3	6.3	7.9	19.4	9.2	20.4	1040.7	47.6	1094.6		Not Processed
209774_x_at	9.6	27.8	30.1	67.6	239.4	704.4	19.7	14.3	10.4	8.1	20.4	14.3	19.6	709.4	14.5	293.8		MIP-2alpha
202688_at	67.2	173.6	21.8	252.6	158.3	295	29.6	15.6	30.2	78.9	22.8	39.5	62.3	114.3	86.3	24.6		TRAIL
210511_s_at	132.1	145.9	1	1037.3	166.4	1808.6	132.1	66.9	101.2	123.5	95.7	96.2	82.3	1487.4	52	861.1		Inhibin-betaA
205890_s_at	38.4	267	36	521.2	160.9	600.1	12.3	39.9	2.8	118.7	66.7	3.5	60.9	315.8	12.1	31.6		Ubiquitin-like protein FAT10
208747_s_at	55.4	128	4	195.3	124.2	302.2	5.2	165.8	3.8	139.6	30.6	1.9	107.5	241.8	19.3	26.5		C1 Esterase
204470_at	6.9	26.4	8.8	20	591.7	1072.7	14.9	7	4.5	9.9	10.2	6.4	7.9	711.3	13.1	159.1		Neutrophil-activating Protein-3
214038_at	9.2	338.2	11.9	282.1	485.2	1894.6	31.1	6.1	14.3	34.5	26.9	5.8	65.1	1217.4	55.8	133.3		MCP-2
204614_at	1.5	27.2	11.5	78.5	85.9	729.5	6.6	13.5	6	13.4	30.1	1.4	8.5	67.5	12.6	207.7		PAI-2
217165_x_at	29.4	205.1	100.6	136.8	650.3	625.7	94.4	83.4	105.5	62.1	56.7	71.6	116.2	314.2	468.5	312.8		Metallothionein-1F
205000_at	118.5	140.5	1.9	203.9	155.5	199.1	1	201.9	0.5	5.6	125.1	5.7	99.8	147.2	84.3	166.5		DEAD-box Protein-3
204409_s_at	112.7	117.2	0.3	196.9	125.8	175.3	0.4	138.2	0.2	0.5	57.6	2.9	80.5	108.9	102.3	107		Eukaryotic Translation Initiation Factor 1A
201909_at	587.1	719.1	12.4	628.1	868.3	670.6	22.9	557.9	15.9	15.8	652.6	17.8	625.2	558.6	545.1	678.4		40S ribosomal protein
216598_s_at	32	1350.5	278	201.3	2186.7	2282.9	292.4	360.9	59.9	119.3	353.3	53.5	406.7	292	155	124.1		MCP-1

	NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
	HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	CD3					
203915_at	241.2	1351.5	86.1	923.2	453.8	2109.4	79.3	89.3	26.5	204	114.6	39.1	279.6	1034.4	40.3	74.9	MIG
204533_at	63.3	2054.2	48.7	1200.1	530.8	1746.8	108.7	46.6	33.3	137.9	67.3	27.8	402.3	528	95.7	69.5	IP-10
203290_at	34.2	105.4	35	27.5	3914.5	110.6	5.4	1569.2	9.5	5.4	715.1	5.8	424.1	1516.2	14.5	3	HLA-DQalpha1
219519_s_at	24	277.3	10.8	54.2	195.9	260.5	2.2	4.4	6	35.6	23.5	4.3	42.5	42.1	110.8	32.5	Sialoadhesin
208075_s_at	36.4	224.4	166.9	81.2	123.4	763.2	120.9	63.6	25.9	45.3	79.9	8.9	51.6	77.6	78.8	54	MCP-3
220655_at	1.5	1.5	1.1	14.6	48.5	31.2	3.7	1.9	5	2.9	2.3	1.1	1.5	35.4	1.9	11.6	Hypothetical Protein
205990_s_at	4.6	5.5	12.1	36	55	28.1	11.3	7.7	5.1	2.6	3	3.3	7.4	50	10.1	46.7	WNT-5A
205767_at	5.2	10.6	6.1	53.5	169	87.2	22.1	5.2	4.9	10.4	9.4	4.8	8.4	234.4	10.9	94.8	Epiregulin
203835_at	5.9	5	2	18.1	54.6	38.6	7	4.3	4.8	4.8	7.7	4.7	16.4	26.2	3.7	48.7	GARP
207533_at	0.9	4	1.2	44.2	72.6	19.8	2.4	4.5	1.8	11.3	2.9	2.3	3.2	16.6	3.2	1.7	I-309
218880_at	11.1	23.5	12	53.1	152	94.9	26.3	20	25.7	12	26.1	26.3	34.9	161.6	27.4	86.1	Hypothetical Protein
210548_at	11.5	6.3	16.4	32	63.9	118.5	5.3	4.2	18.9	4.3	21	20.7	13.5	64.5	8.8	76.3	HMRP-2A
204698_at	1.2	4.9	5.9	16.6	114.6	43.9	2.5	1.4	1.3	2.7	2.2	1.2	1.9	44	3.5	10.8	ISG20
204879_at	1.8	2.6	1.1	7.4	72.8	76.5	44.9	4	8	1.5	3.6	15.7	2.1	10.8	29.9	111.6	T1A-2
41577_at	14.5	34.1	4	40	73.3	62.8	4.7	49.6	2.3	19.3	4.5	2.6	15.4	37.2	4.4	14.5	Hypothetical Protein
204156_at	22.8	26.1	2.4	45.8	58.6	73.2	26.5	25.3	14.6	23.3	2.1	26.7	15.9	55.8	16.9	40.7	EST
222326_at	4.5	14.9	11.2	18.2	105.2	57.6	7.7	10.1	5.9	7.2	14.9	7.2	8.3	99.1	5.9	45.7	EST
212823_s_at	7.3	41.9	14.2	39.9	69.1	101.6	19.1	32.8	24	26.7	5.5	30.1	32	52.6	5.2	42.9	Hypothetical Protein
206148_at	11	36.4	4.1	32.1	109	50.5	3.7	4.4	3.6	17.7	25.3	3.1	13.4	33.5	3.4	5.8	IL-3RA
209795_at	18.5	40.1	33.6	39.6	56.5	98.3	15.7	37.2	13.1	16.4	7.8	19.2	30.5	46.3	20.2	17.1	CD69
215101_s_at	0.8	0.3	0.3	5.9	782.3	183.7	14.9	0.9	0.3	0.5	8.5	0.6	1	762.5	8.6	857.4	ENA-78
210773_s_at	19.8	35.3	15.1	47.6	129.3	40.7	18.4	16.2	18.5	26.7	11.7	9.9	14	101	25.4	96.5	Lipoxin A4R
203828_s_at	21.8	80.4	5.7	52.2	174.3	119.6	5.2	31.5	1.5	19.9	6.9	5.2	45.7	164.4	3.2	7.9	NK4
209339_at	35.2	75.1	22	62.6	81	95.4	72.1	57.2	56.7	14.5	6.4	40.7	72.1	88.5	43.3	44.5	hSIAH2
219423_x_at	10.5	28.5	28	21	60.7	59.6	24	29.1	13.6	18.2	5.4	6	35.8	31.9	21	21.4	TNFRSF12
218983_at	54.7	107.2	8.7	192.7	195.1	75.9	16.4	57.5	20.8	29.6	15.6	29.5	65.8	150.6	26	65.1	Complement C1r-like proteinase
218995_s_at	11.8	39.2	2.7	139.8	563.8	32.6	22.1	1.3	7.5	30	18.9	13.5	15.7	252.8	20.8	310.9	Endothelin-1
205146_x_at	4.5	29	1.7	19.8	31.4	36	7.3	8.5	21.1	3.2	3.7	23.2	29.8	20.2	6	22.1	APB-A3
204439_at	6	64.6	2.2	45.9	74.3	48.9	4.4	2.2	1.3	11.8	16.9	1.3	7.8	27.9	135.1	28.7	Hypothetical Protein
205001_s_at	32.1	44.6	5.4	40.8	66.4	56.3	3.8	41.7	6.4	1.9	30.3	1.7	45.2	37.8	28	46.3	Dead box, Y isoform
213173_at	20.6	19.5	1.9	27.1	42.5	24.8	20	18.9	15.1	15.4	1.3	16.8	18.8	34.7	15.2	30.1	Hypothetical Protein
209304_x_at	19.7	52.7	45.8	48.2	54.5	90.3	6.8	10.3	13.9	49.1	10.4	3.7	26.1	51.6	7.5	28.6	GADD45B
206187_at	4.3	25.9	19.5	13.3	41.1	82.6	24.6	18.2	28.3	5.8	9	12.6	15.4	31	16.8	35.2	Prostacyclin Receptor
216614_at	10.1	49.1	10.6	22.8	50.4	116.1	24	17.4	29	3.5	13.4	20.3	28.8	38.7	16.1	29.9	EST
209583_s_at	23.6	20.6	19.4	24.7	23.8	51.1	13.8	32	2.3	12.5	6.8	4.1	15.6	9.4	13.2	8.3	my033
204166_at	5.1	61.6	6.7	22.8	82.2	63.2	11.6	1	29.8	26.4	3.7	16.6	28.3	30.6	25.3	44.4	Hypothetical Protein

	NIL				MDP			NIL			MDP		NIL	MDP	NIL	MDP	
	HC1	HC2	HC3	HG1	HG2	HG3	CD1	CD2	CD3	CD4	CD5	CD6					
210229_s_at	12.5	40.3	3.3	36.7	116.9	23.2	3.7	12.8	1.7	53	5.4	2.3	25.9	47.6	6.6	2.4	GM-CSF
210354_at	31.8	95.1	21.3	139.2	299.6	41.8	19	31.1	8.6	47.8	21.5	13	27	217.5	17.3	22	INF-gamma
207072_at	16.5	44.8	64.3	37.6	86.2	133.9	13.2	34.8	8.7	13	16.2	28.9	31.2	57.1	16.8	28.2	IL-18R-associated Protein
206341_at	11.1	38.6	1.4	52.5	141.6	13.7	7.6	9.4	0.9	21.8	11.6	4.5	7.7	97.4	6.8	5.4	IL-2R
214329_x_at	8.9	47.5	10.3	73.6	69.5	18.1	11.6	8.7	9	5.1	0.6	16.7	9.1	39.6	27.3	9.7	EST
205207_at	18.5	27.2	19.1	25.2	418.4	45.7	19.5	16.9	15.9	24.2	14	9.9	18.4	378.1	18.7	27.2	IL-6
203249_at	55.9	46.9	22.6	57.1	43.5	77.9	34.7	52.2	42.9	5.1	5.3	62	47.7	37.3	38.5	51	EST
212999_x_at	321.7	292.1	268.5	339	284.7	18.1	124.8	15.8	1	222	2.4	13.7	152.8	261.6	35.4	38.7	HLA-DQbetaII
221807_s_at	6.7	50.8	9.5	28.6	38.2	25.8	19.4	15.7	23.6	5.6	4.2	19.9	45.1	34.4	25.7	50.7	Hypothetical Protein
204410_at	81.6	63.7	3.7	88.7	70	56.8	2	64.2	4.4	0.5	41.8	1.7	41.8	59.9	49.3	63	Translation initiation factor 1A
202661_at	8.2	35.9	26.9	19.7	28	47.9	25.1	31	31.8	2.3	2.1	41.6	27.9	36.6	16.9	28.3	Inositol 1,4,5-Triphosphate Receptor-2
214131_at	23.7	41.6	8.8	19.5	37.6	42	7.4	31.8	5.5	1.9	22.2	5.3	22.2	21.8	19.7	38.6	EST
209392_at	55.4	121.5	21.6	47.4	88.6	130.9	69.3	77.1	8.6	17.4	34	10	36.5	58.8	40	35.6	Autotaxin
210116_at	21.5	40.3	24.7	21.4	40.4	40.3	7.3	30.8	6	19.5	2.6	6.2	17.3	18.4	6.2	4.8	T cell signal transduction molecule SAP
214567_s_at	6.9	74.7	38.6	16.1	42.2	227.7	1.8	24.1	2.7	39.6	2.1	12.6	9.6	1.3	8.5	2.8	SCY-C2
217584_at	33.1	40	15.4	38	43.9	24.6	4.9	18.4	36.7	6	3.6	25	25.8	31.6	22.4	26.8	EST
213915_at	18.2	101.6	65.1	21.2	126.5	128.7	1.9	27.1	17.5	24.4	3.4	14	25.9	31.7	19.9	19.4	NKG7
211122_s_at	4.9	109.2	9.8	41.1	85.2	9.2	1.6	6.2	2.1	8.8	1	5.9	7.8	11.2	6.1	7.1	H174
214450_at	2	92.9	61.5	1.7	84.4	162.1	7.5	40.1	7.5	6.1	7.9	6.3	37.3	27.8	21.5	14.8	Cathepsin W
220005_at	26.2	67.2	13.9	44.7	24.8	40.7	3.3	27.1	0.9	1.8	8.3	7.7	17.5	0.9	1.7	2	GPR86
219725_at	1307.7	1026.1	1584.2	534.7	254.3	259.9	1110.3	872.3	1006.8	769.6	1441.8	951.7	940.8	540.7	982.9	749.2	TREM-2
220380_at	298.5	161.4	256.8	137.4	42	5.7	147.9	374	324.4	139.3	436.3	336	307.9	197.9	325.2	149.2	DNAse-IIbeta
204141_at	506.1	250.6	605.1	224.4	114.1	45.6	559.5	1005.7	560.6	420.5	394.1	527.3	536.1	357.5	571.3	399.7	Tubulin-beta
201005_at	1250.6	654.6	1260.4	684.3	247.9	199	1014.6	572.9	1630.8	899.3	905.6	1321.4	1087.1	684.1	1084.8	861.7	CD9
202741_at	822.2	480.1	939.7	493.4	226.5	147.8	719.5	1047.2	772.2	673.2	1033.1	903.1	718.3	452.7	709.6	457.5	EST
219045_at	265.5	182	270.2	166	156.1	86.1	247.2	218.2	274.9	647.8	600.8	256.4	258	170.8	285	225.3	Hypothetical Protein
212949_at	902.3	394.1	400.9	454.1	123.8	3.4	558.8	665.8	689.2	631.4	1300.6	564.1	677.6	271.9	627.6	310.5	HCAP-H
201125_s_at	493.3	229.5	669.2	286.9	127.6	46.5	449.9	372.5	450.6	565.1	540.9	445.6	350	263.6	346.5	293.2	Integrin-beta5
212552_at	1075.1	857.5	1845.4	452.3	531.4	251.7	965.5	1615.2	1171.7	1125.2	1312.7	1100.3	1323.7	856.9	1316.2	985.6	Hippocalcin-like-1
217963_s_at	298.7	88.3	139.3	95.5	72.3	22.1	527.1	143.9	690.2	294.9	155.6	636	304.6	274	404.1	599.9	p75NTR-associated Cell Death Executor
210136_at	175.7	292	462.1	97.8	183.9	61.9	112.8	288.9	384.3	323.2	534.6	484.9	296.3	123.5	227.8	268.3	Myelin Basic Protein
203980_at	974.3	568.4	3451.8	502.9	75.3	10.4	1299.9	2373	92.7	1936.4	1533.4	88.6	1869.7	1479.3	1433.4	998.3	FABP4
201540_at	247.6	105.1	578.1	107.9	47.6	39.5	304.1	346.8	297.5	190	210.9	319.2	202.2	122.8	268.2	168.8	FHL-1
204580_at	343.2	167.4	305.6	287	83.7	27.2	664.6	1691.9	66.7	299.6	955.6	85.7	818.5	914.1	277.5	335.9	MMP12
208423_s_at	203.3	230.7	563.9	155.7	153.7	37.9	154.8	197.8	189.2	280.4	644.6	237.5	134.5	122.9	218.3	248.4	MSR1
202575_at	1130.5	793.6	274.3	631.4	284.1	12.9	731	422.4	1788	617.2	486.7	1528.5	795.6	579.8	827.7	734.1	CRABP2

	NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
	HC1	HC2	HC3	MDC1	MDC2	MDC3	CD1	CD2	CD3	CD1	CD2	CD3					
220475_at	139.3	128.5	115.6	91.3	114.1	22.1	125.3	95.7	180.2	234.4	295.7	225.8	229.3	134	218.3	205.7	CNT3
211887_x_at	268.5	238.1	739.8	215.6	197.4	41.5	194.9	255.2	275.1	352.7	708.4	315.8	173.2	166.7	281.2	370.2	SR-A
218856_at	624.7	170.3	189.6	336	67.8	9.8	304.5	184.9	317.7	244.5	219.2	265.2	207.8	125.8	183.6	111.3	Hypothetical Protein
218885_s_at	652.7	278.8	249.5	491.3	117.2	14.3	459.9	550.9	502.3	340.7	246.7	498.5	577.4	288.9	608.4	276.2	Hypothetical Protein
204430_s_at	444	214.2	106.9	264.6	63	2.6	518	329.4	114.8	320.7	464.6	123.5	270.7	181.1	232.2	140.6	SCL2A5
211470_s_at	149.2	98.1	91.8	102.6	78.2	5.7	60.5	120.1	38.2	250.2	280.2	55.5	83.6	76.9	191.9	131.3	SULT1C1
208168_s_at	1806.5	1206.7	871.4	2056.3	709.1	3.4	119.2	1114	1181.8	662.6	4793.7	961.4	1301	617.2	1256.6	901.5	Chitinase-1
210138_at	214	63.7	63.5	131.9	30.1	6.3	78.3	162.5	154.5	126	163.5	142.7	149.4	69.2	139.1	97.5	RGSZ1
202450_s_at	707.3	424.5	213.5	474	316.4	96.5	400.8	693.3	835.5	864.9	1673.1	795.5	1443.5	1203.6	1349.6	1119.9	Cathepsin K
221728_x_at	9	25.8	557.6	5.3	15.4	16.8	617.9	8.7	645	470.1	31.3	582.6	161.6	127.8	221.8	108.3	EST
204517_at	226.8	118.5	71.3	208.3	82.4	1.7	96.4	202.6	187.4	164	179.5	165.7	165.5	144.2	200	203.3	Cyclophilin C
215856_at	82.6	48.1	80.5	128.9	56.7	15	52.7	194.4	220.4	131.5	345.2	269	203.8	191.3	137.3	151.9	EST
211748_x_at	315.3	270.9	67	245.2	249.5	21.1	72.4	190.4	772.8	580.1	235.1	476.3	527	361	416.4	495.2	Prostaglandin D2 Synthase
206881_s_at	3.8	69.8	255.7	2.8	217	47.5	193	478.4	380.1	306.8	452.8	381.2	460.6	370.5	360.6	240.1	LILRA3
219225_at	161.8	25.9	54.8	80.3	67	3	165.7	49.4	64.7	159.4	87.9	76.8	70.5	61.3	94.5	64.7	Hypothetical Protein
201058_s_at	149.7	22.8	96	78.4	110.7	1.9	60.9	206.5	103.6	58.9	232.8	76.9	192	109.7	146.1	91.6	MRLC-2
202134_s_at	134.1	21.9	66.3	111.6	53.5	3.1	45.8	93.9	114.9	119.8	133.3	98.5	88.6	85.2	107.3	68.3	TAZ
201904_s_at	93.9	19.3	18.1	18.8	32.1	4.6	14.8	46.7	101.5	62.8	86.3	91.7	89.8	26.3	55.8	28.5	HYA22
201474_s_at	81.3	16.7	108.9	38.4	75.2	3.4	119.7	72.2	157.5	71.1	126.3	129.8	152.2	69.9	91.4	76.8	CD49C
219263_at	67.9	21.2	20	52.9	27.3	0.3	96	46.2	82.8	57.9	64.4	68.3	63.7	53.4	59.4	69.3	Hypothetical Protein
211126_s_at	64.7	7.3	11.9	38.6	47.8	2.6	38.2	38.9	53.9	45.1	72.4	48.1	76.7	44.2	76.5	84.7	h-SmLIM
204412_s_at	62.6	16.2	39.1	10.7	35.6	16.1	69.5	103	58.4	93.5	114.8	57.8	10.5	49.6	93.8	70.3	Neurofilament, Heavy Polypeptide
210402_at	53.1	18.9	4.3	12.9	38	1.8	26.7	66.6	59.4	33.6	34.8	61.7	35.6	25.7	164.7	67.3	ROMK1
201905_s_at	52.1	13.3	17	32.1	28.1	1.9	36.8	50.3	31.2	34.6	65.5	41.3	37.3	29.6	39.1	14.1	HYA22
204083_s_at	50.1	4.7	24.4	17.5	24.4	3.9	109	34.9	28	41.3	44.2	24.2	43.6	33.2	35.2	14.9	Tropomyosin-2
216604_s_at	49.2	73.9	204.6	26.6	69.1	2.2	56.3	43.4	62.5	117.2	136.6	62.6	130.3	36.3	93.4	141.4	SCL7A8
214040_s_at	30.1	25.5	40.1	3.7	58.5	39.5	59.5	3.8	110.8	156.8	104.3	95.6	51.8	23.2	34.3	44.8	Gelsolin
216603_at	29.7	48.4	73.7	11.8	49.7	2	25.7	12.5	41.7	65.9	108.9	37.6	63	19.1	45.3	81.8	SCL7A8
210298_x_at	29.1	3.7	74.2	13.1	19.7	2.3	58.8	29.9	63.1	22.1	32.7	76	34.7	14.7	54.8	35.1	FHL1
208422_at	26.8	22.6	339	16.6	18.9	1.8	18.3	63.9	22.3	35.4	274	20.9	28.1	19.3	22.5	27.8	MSR1
201015_s_at	24.5	2.4	2.7	6.2	27.9	44.6	21.5	25.7	64.2	58.5	51	57.3	47	20.4	39.8	19.3	Plakoglobin
215382_x_at	23.8	9.6	3.6	14.6	33.1	9.6	878.1	9	14.8	234.5	54.5	13.4	48.5	72.1	32.6	15.8	Tryptase
210084_x_at	13.1	3.7	4.2	6.3	5	2.7	872.6	3.7	3.6	233.5	47.4	5.1	51.8	68.8	14.5	5.6	Tryptase
202112_at	12.6	8.4	32.3	4.8	18.8	5.7	20.3	60.4	39.8	27.6	54.9	32.2	26.8	13.8	42.7	22.5	VWF
219271_at	9.8	3.7	14.1	3.3	5	2.6	9.7	43.6	29.7	22.4	57.8	20.5	34.3	16.2	24.3	20.6	Hypothetical Protein
203496_s_at	8.5	31.4	83.5	1	32.9	32.8	5.4	26.8	24.9	66.5	97.3	46.5	39.9	15.8	32.6	46.4	TRAP220

	NIL			MDP			NIL				MDP		NIL	MDP	NIL	MDP	
	HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD5	CD7	CD2	HCD5	Ileal	Ileal	Colonic	Colonic	
214218_s_at	7.6	12.1	368	14.9	13.9	6.9	313.1	11.6	287.2	251.3	11.9	329.4	101.4	80.3	129.3	53.2	EST
204163_at	6.4	31.3	8.3	3.6	28.1	5.3	7.2	2.2	38.6	75.5	62.7	36.8	23.3	13.6	19.7	49.8	EMILIN
203215_s_at	3.5	2.6	29.6	4.6	8.7	1.8	3.1	11.5	18.5	35.3	40.2	22.8	19.1	2.2	5.9	9.2	Myosin VI

Table A2.2: Normalised and Scaled Data

NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	CD3					Description
-1.051	-0.767	-0.917	1.122	1.135	1.277	-1.912	-0.254	-1.069	-0.025	-0.469	-2.138	-1.659	1.032	-0.501	0.81	MIP-1delta
0.04	0.358	-0.411	2.076	2.51	2.198	0.54	-0.011	0.552	0.911	0.315	0.571	0.457	2.453	0.32	2.482	TSG-6
0.512	0.723	-0.395	2.097	2.519	2.316	1.006	0.525	1.002	1.377	0.63	0.998	0.8	2.37	0.719	2.448	TSG-6
-0.099	0.922	0.248	2.341	2.821	2.89	0.785	1.136	0.861	1.808	1.883	1.036	0.991	2.522	1.31	2.764	IL-8
0.307	0.989	0.897	2.425	3.02	3.088	1.221	0.694	0.671	1.681	2.154	0.605	0.841	2.665	0.937	2.808	IL-1beta
0.262	0.951	0.591	2.204	2.878	2.788	1.048	0.733	0.3	1.163	1.677	0.64	0.656	2.514	0.911	2.653	IL-1beta
-0.35	0.75	0.039	1.365	1.655	1.684	0.368	0.553	-0.009	0.571	0.191	0.026	0.424	1.79	0.209	1.414	PDE-5
-1.462	-0.163	-1.997	0.919	0.847	1.475	-1.54	-1.363	-1.809	-0.146	-0.606	-1.709	-1.144	0.463	0.013	0.392	EBI-3
-0.041	0.361	0.002	1.046	1.755	1.442	-0.137	0.09	0.056	0.597	0.395	0.113	0.211	1.539	0.066	1.164	Hypothetical Protein
-0.334	0.016	-0.57	1.637	2.643	2.665	-0.51	0.077	0.196	0.545	0.636	0.127	0.126	2.334	0.507	2.421	IL-8
0.752	2.054	-0.432	2.453	2.831	2.744	0.576	0.384	-0.336	1.974	0.775	-0.387	0.79	2.471	0.184	1.511	IDO
-0.266	0.591	-0.012	0.848	1.359	1.201	0.119	0.125	-0.273	0.155	0.203	-0.303	0.077	1.405	-0.135	0.893	PDE-5
0.939	1.823	0.788	2.038	2.297	2.304	0.907	0.906	0.643	1.495	0.656	0.718	1.193	1.926	0.67	1.853	GTP Cyclohydrolase
-0.354	0.59	-1.856	1.097	1.096	1.644	0.033	0.112	-0.071	-0.175	-0.418	0.032	-0.09	0.556	0.259	0.407	TSC403 protein
-1.078	-0.202	-1.473	1.768	2.331	1.025	-1.814	-0.225	-1.394	-0.004	-1.127	-1.103	-1.032	2.326	-0.978	2.122	MIP-3alpha
-0.567	-0.16	-0.194	0.578	1.719	1.647	-0.137	-0.468	-0.347	-1.363	0.06	-0.765	-0.393	1.724	-0.206	0.915	IL-1alpha
0.573	1.416	0.05	1.558	2.154	1.929	-0.242	0.501	-0.207	0.886	0.437	-0.071	0.693	1.667	-0.068	1.163	CD38
-1.737	-0.914	-0.909	0.424	1.845	1.153	-0.639	-0.645	-1.598	-0.878	-0.596	-1.186	-0.898	1.842	-0.538	1.446	GRO-3
0.231	0.812	-0.387	1.71	1.779	0.901	-0.235	-0.082	-0.148	1.144	0.21	-0.608	0.511	1.246	-0.102	0.455	HM74
0.166	0.677	-1.086	0.761	1.314	1.019	-0.534	0.13	-0.085	0.464	-0.586	-0.296	0.163	0.927	0.102	0.587	Properdin
-0.29	-0.468	-0.628	1.379	1.788	0.228	-0.278	-0.416	-0.768	-0.91	-0.355	-0.224	-0.365	2.038	-0.425	1.841	COX-2
-1.362	-0.367	-1.593	0.317	2.407	1.897	0.907	-0.645	-0.827	-0.962	-0.426	-0.701	-0.171	2.07	0.321	2.244	Not Processed
-0.59	-0.011	-0.023	0.522	1.983	1.37	-0.309	-0.341	-0.546	-0.945	-0.386	-0.428	-0.193	1.852	-0.346	1.432	MIP-2alpha
0.526	1.068	-0.249	1.238	1.441	1.099	-0.024	-0.292	0.054	0.674	-0.297	0.2	0.466	0.811	0.654	-0.1	TRAIL
0.914	0.966	-2.408	2.005	2.57	1.132	1.024	0.522	0.733	0.993	0.854	0.75	0.625	2.274	0.37	2.096	Inhibin-betaA
0.205	1.322	0.102	1.631	1.884	1.11	-0.639	0.233	-1.283	0.965	0.564	-1.298	0.453	1.39	-0.447	0.054	Ubiquitin-like protein FAT10
0.415	0.888	-1.437	1.098	1.456	0.94	-1.243	1.03	-1.112	1.08	-0.061	-1.676	0.777	1.238	-0.185	-0.054	C1 Esterase
-0.78	-0.042	-0.885	-0.14	2.245	1.962	-0.505	-0.741	-1.017	-0.802	-0.942	-0.925	-0.712	1.853	-0.403	1.053	Neutrophil-activating Protein-3
-0.615	1.461	-0.673	1.298	2.599	1.832	0.011	-0.818	-0.367	0.086	-0.164	-0.986	0.491	2.16	0.41	0.943	MCP-2
-1.655	-0.024	-0.697	0.603	2.005	0.699	-1.076	-0.373	-0.855	-0.587	-0.074	-1.865	-0.67	0.51	-0.425	1.217	PAI-2
0.052	1.166	0.822	0.905	1.91	2.023	0.789	0.646	0.757	0.504	0.434	0.567	0.822	1.387	1.603	1.47	Metallothionein-1F
0.851	0.943	-1.959	1.122	1.197	1.087	-2.398	1.141	-2.252	-1.207	1.068	-0.997	0.735	0.955	0.641	1.081	DEAD-box Protein-3
0.822	0.836	-3.252	1.103	1.117	0.949	-3.04	0.928	-2.767	-2.925	0.446	-1.415	0.612	0.783	0.75	0.808	Eukaryotic Translation Initiation Factor 1A
1.769	1.905	-0.644	1.733	1.953	2.212	-0.204	1.709	-0.307	-0.469	2.393	-0.293	1.781	1.715	1.688	1.949	40S ribosomal protein
0.1	2.276	1.534	1.115	2.715	2.816	1.581	1.466	0.439	0.968	1.901	0.387	1.536	1.346	0.983	0.899	MCP-1

NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	CD3					
1.259	2.277	0.713	1.942	2.666	1.788	0.667	0.684	-0.02	1.35	0.998	0.193	1.322	2.067	0.227	0.587	MIG
0.492	2.524	0.314	2.085	2.549	1.89	0.888	0.32	0.109	1.072	0.571	-0.017	1.53	1.683	0.712	0.541	IP-10
0.138	0.774	0.082	0.033	0.831	3.197	-1.216	2.288	-0.597	-1.233	2.467	-0.986	1.56	2.285	-0.346	-1.4	HLA-DQalpha1
-0.065	1.344	-0.741	0.402	1.364	1.239	-1.845	-1	-0.855	0.108	-0.273	-1.171	0.248	0.241	0.795	0.072	Sialoadhesin
0.174	1.219	1.177	0.621	2.033	0.936	0.962	0.494	-0.033	0.28	0.709	-0.721	0.359	0.59	0.603	0.385	MCP-3
-1.655	-1.731	-2.342	-0.311	0.317	0.037	-1.481	-1.47	-0.957	-1.675	-2.137	-2.014	-1.659	0.142	-1.486	-0.565	Hypothetical Protein
-1.012	-0.966	-0.662	0.18	0.396	-0.032	-0.699	-0.687	-0.946	-1.753	-1.924	-1.335	-0.749	0.339	-0.549	0.296	WNT-5A
-0.942	-0.579	-1.141	0.395	1.095	0.709	-0.229	-0.907	-0.969	-0.767	-1.008	-1.103	-0.677	1.22	-0.506	0.733	Epiregulin
-0.87	-1.022	-1.923	-0.194	0.391	0.176	-1.034	-1.013	-0.98	-1.317	-1.168	-1.116	-0.295	-0.029	-1.112	0.322	GARP
-1.948	-1.153	-2.281	0.291	0.568	-0.26	-1.784	-0.988	-1.532	-0.708	-1.951	-1.558	-1.227	-0.289	-1.193	-1.751	I-309
-0.507	-0.11	-0.667	0.391	1.029	0.764	-0.107	-0.153	-0.037	-0.665	-0.189	-0.052	0.136	1.008	0.011	0.673	Hypothetical Protein
-0.487	-0.886	-0.449	0.116	0.489	0.91	-1.229	-1.026	-0.21	-1.395	-0.363	-0.2	-0.406	0.485	-0.626	0.599	HMRP-2A
-1.783	-1.034	-1.165	-0.241	0.853	0.26	-1.756	-1.641	-1.715	-1.726	-2.172	-1.96	-1.524	0.266	-1.143	-0.609	ISG20
-1.55	-1.407	-2.342	-0.68	0.57	0.624	0.268	-1.054	-0.693	-2.144	-1.777	-0.371	-1.467	-0.534	0.06	0.834	T1A-2
-0.354	0.109	-1.437	0.237	0.574	0.494	-1.313	0.355	-1.394	-0.327	-1.598	-1.482	-0.331	0.171	-1.015	-0.427	Hypothetical Protein
-0.094	-0.048	-1.795	0.31	0.435	0.595	-0.102	-0.022	-0.355	-0.193	-2.21	-0.042	-0.313	0.402	-0.26	0.211	EST
-1.025	-0.379	-0.716	-0.191	0.799	0.438	-0.968	-0.535	-0.864	-1.028	-0.638	-0.852	-0.683	0.729	-0.85	0.282	EST
-0.747	0.231	-0.55	0.235	0.538	0.809	-0.331	0.124	-0.076	-0.096	-1.437	0.032	0.086	0.368	-0.921	0.243	Hypothetical Protein
-0.512	0.148	-1.42	0.117	0.821	0.352	-1.481	-1	-1.142	-0.389	-0.214	-1.373	-0.41	0.111	-1.159	-0.993	IL-3RA
-0.214	0.205	0.054	0.231	0.412	0.788	-0.468	0.194	-0.416	-0.443	-1.157	-0.246	0.059	0.295	-0.16	-0.325	CD69
-2.016	-2.679	-3.252	-0.803	2.049	1.196	-0.505	-1.888	-2.539	-2.925	-1.088	-2.389	-1.89	1.893	-0.639	2.093	ENA-78
-0.175	0.13	-0.506	0.331	0.928	0.211	-0.357	-0.271	-0.222	-0.096	-0.832	-0.656	-0.385	0.74	-0.031	0.744	Lipoxin A4R
-0.12	0.614	-1.189	0.381	1.114	0.916	-1.243	0.101	-1.634	-0.305	-1.256	-1.054	0.289	1.018	-1.193	-0.802	NK4
0.155	0.574	-0.243	0.48	0.637	0.768	0.6	0.435	0.408	-0.53	-1.316	0.218	0.549	0.665	0.268	0.266	hSIAH2
-0.539	0.004	-0.074	-0.113	0.457	0.46	-0.171	0.057	-0.395	-0.369	-1.452	-0.965	0.15	0.083	-0.138	-0.186	TNFRSF12
0.408	0.784	-0.893	1.091	1.184	0.618	-0.438	0.438	-0.156	-0.023	-0.601	0.019	0.497	0.968	-0.018	0.501	Complement C1r-like proteinase
-0.472	0.191	-1.712	0.917	1.845	0.066	-0.229	-1.683	-0.729	-0.013	-0.447	-0.464	-0.32	1.263	-0.143	1.467	Endothelin-1
-1.025	0.014	-2.037	-0.145	0.047	0.131	-1.005	-0.632	-0.148	-1.605	-1.755	-0.129	0.046	-0.177	-0.841	-0.167	APB-A3
-0.86	0.486	-1.856	0.312	0.583	0.331	-1.36	-1.388	-1.715	-0.677	-0.537	-1.911	-0.719	0.007	0.906	-0.005	Hypothetical Protein
0.102	0.267	-1.227	0.248	0.513	0.423	-1.462	0.258	-0.819	-1.976	-0.069	-1.745	0.283	0.18	0.023	0.29	Dead box, Y isoform
-0.152	-0.22	-1.959	0.025	0.235	-0.113	-0.299	-0.185	-0.336	-0.488	-2.594	-0.329	-0.217	0.131	-0.319	0.024	Hypothetical Protein
-0.178	0.366	0.271	0.338	0.39	0.732	-1.055	-0.524	-0.383	0.337	-0.927	-1.264	-0.03	0.357	-0.716	-0.007	GADD45B
-1.051	-0.053	-0.327	-0.361	0.214	0.674	-0.154	-0.206	0.017	-1.182	-1.042	-0.507	-0.331	0.067	-0.263	0.121	Prostacyclin Receptor
-0.561	0.324	-0.754	-0.069	0.341	0.896	-0.171	-0.231	0.031	-1.541	-0.723	-0.212	0.026	0.193	-0.287	0.02	EST
-0.074	-0.188	-0.331	-0.025	-0.126	0.36	-0.559	0.11	-1.394	-0.636	-1.267	-1.201	-0.324	-0.614	-0.398	-0.771	my033
-0.953	0.458	-1.076	-0.069	0.646	0.499	-0.68	-1.829	0.046	-0.104	-1.755	-0.336	0.016	0.059	-0.034	0.264	Hypothetical Protein

NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	CD3					
-0.439	0.208	-1.572	0.19	0.865	-0.157	-1.481	-0.403	-1.564	0.391	-1.452	-1.558	-0.034	0.311	-0.787	-1.538	GM-CSF
0.097	0.713	-0.265	0.914	1.451	0.228	-0.335	0.094	-0.652	0.318	-0.344	-0.487	-0.011	1.178	-0.247	-0.169	INF-gamma
-0.28	0.27	0.509	0.203	0.675	0.99	-0.59	0.157	-0.646	-0.608	-0.571	0.007	0.072	0.415	-0.263	-0.016	IL-18R-associated Protein
-0.507	0.182	-2.173	0.385	0.984	-0.501	-0.977	-0.576	-1.921	-0.24	-0.839	-1.143	-0.726	0.72	-0.77	-1.037	IL-2R
-0.634	0.304	-0.774	0.568	0.541	-0.319	-0.68	-0.619	-0.627	-1.274	-3.214	-0.332	-0.631	0.206	0.009	-0.675	EST
-0.214	-0.024	-0.342	-0.014	1.659	0.287	-0.316	-0.247	-0.307	-0.166	-0.688	-0.656	-0.229	1.493	-0.203	-0.038	IL-6
0.42	0.297	-0.224	0.43	0.25	0.635	0.087	0.384	0.251	-1.274	-1.467	0.478	0.314	0.172	0.202	0.35	EST
1.424	1.374	1.51	1.398	1.419	-0.319	0.984	-0.285	-1.862	1.41	-2.103	-0.455	0.978	1.283	0.155	0.18	HLA-DQbetaII
-0.797	0.344	-0.831	0.055	0.169	-0.087	-0.32	-0.289	-0.085	-1.207	-1.654	-0.224	0.282	0.126	-0.025	0.346	*Hypothetical Protein*
0.637	0.477	-1.492	0.669	0.546	0.429	-1.912	0.499	-1.029	-2.925	0.189	-1.745	0.239	0.442	0.34	0.481	Translation initiation factor 1A
-0.681	0.139	-0.102	-0.148	-0.025	0.317	-0.14	0.092	0.083	-1.84	-2.21	0.232	0.008	0.161	-0.26	-0.014	Inositol 1,4,5-Triphosphate Receptor-2
-0.072	0.226	-0.885	-0.153	0.159	0.231	-0.995	0.106	-0.904	-1.976	-0.318	-1.042	-0.122	-0.134	-0.174	0.178	EST
0.415	0.858	-0.256	0.329	0.692	0.975	0.572	0.602	-0.652	-0.401	0.024	-0.649	0.161	0.432	0.223	0.128	Autotaxin
-0.128	0.208	-0.162	-0.103	0.204	0.204	-1.005	0.088	-0.855	-0.32	-2.038	-0.945	-0.265	-0.231	-0.822	-1.11	T cell signal transduction molecule SAP
-0.78	0.571	0.151	-0.258	0.231	1.337	-1.986	-0.049	-1.304	0.184	-2.21	-0.507	-0.6	-1.742	-0.645	-1.443	SCY-C2
0.12	0.203	-0.493	0.209	0.255	-0.119	-1.284	-0.2	0.163	-1.158	-1.777	-0.083	-0.037	0.078	-0.102	-0.047	EST
-0.223	0.752	0.517	-0.108	0.914	0.964	-1.948	0.017	-0.253	-0.16	-1.823	-0.441	-0.034	0.079	-0.168	-0.247	NKG7
-0.976	0.795	-0.809	0.252	0.668	-0.762	-2.069	-0.809	-1.445	-0.886	-2.805	-0.976	-0.719	-0.514	-0.831	-0.868	H174
-1.49	0.7	0.477	-1.479	0.662	1.115	-0.986	0.236	-0.729	-1.146	-1.147	-0.935	0.174	0.005	-0.125	-0.414	Cathepsin W
-0.014	0.509	-0.564	0.297	-0.1	0.211	-1.561	0.017	-1.921	-2.014	-1.107	-0.811	-0.258	-1.951	-1.548	-1.65	GPR86
2.229	2.115	2.753	1.645	1.349	1.423	2.516	1.959	2.025	2.294	3.029	2.167	2.014	1.697	2.019	2.01	TREM-2
1.381	1.025	1.479	0.907	0.228	-1.075	1.103	1.486	1.388	1.079	2.07	1.523	1.377	1.124	1.398	1.013	DNAse-IIbeta
1.684	1.284	2.079	1.174	0.85	0.285	2.036	2.039	1.696	1.864	1.989	1.802	1.693	1.461	1.714	1.622	Tubulin-beta
2.203	1.85	2.593	1.779	1.333	1.249	2.453	1.724	2.296	2.405	2.656	2.37	2.097	1.831	2.074	2.096	CD9
1.962	1.667	2.387	1.602	1.277	1.054	2.212	2.062	1.876	2.199	2.762	2.134	1.86	1.596	1.836	1.705	EST
1.314	1.096	1.514	1.01	1.045	0.701	1.463	1.184	1.295	2.172	2.327	1.356	1.276	1.04	1.324	1.268	Hypothetical Protein
2.016	1.551	1.791	1.557	0.901	-1.413	2.035	1.808	1.812	2.154	2.946	1.843	1.827	1.305	1.767	1.466	HCAP-H
1.669	1.232	2.15	1.307	0.92	0.298	1.883	1.483	1.573	2.075	2.243	1.698	1.45	1.287	1.434	1.43	Integrin-beta5
2.116	2.009	2.86	1.554	1.808	1.402	2.418	2.304	2.11	2.565	2.954	2.256	2.209	1.959	2.182	2.179	Hippocalcin-like-1
1.382	0.67	1.05	0.71	0.566	-0.189	1.994	0.951	1.813	1.612	1.243	1.918	1.371	1.309	1.52	1.873	p75NTR-associated Cell Death Executor
1.077	1.374	1.89	0.722	1.147	0.485	0.913	1.341	1.484	1.677	2.233	1.75	1.355	0.855	1.199	1.376	Myelin Basic Protein
2.06	1.767	3.299	1.612	0.591	-0.682	2.626	2.519	0.684	2.951	3.078	0.699	2.406	2.271	2.23	2.187	FABP4
1.274	0.772	2.047	0.776	0.306	0.191	1.608	1.443	1.34	1.299	1.487	1.491	1.137	0.852	1.29	1.089	FHL-1
1.461	1.047	1.601	1.307	0.657	-0.053	2.156	2.33	0.499	1.623	2.699	0.679	1.935	1.996	1.309	1.514	MMP12
1.161	1.235	2.03	0.975	1.035	0.164	1.135	1.129	1.085	1.576	2.383	1.309	0.905	0.852	1.175	1.328	MSR1
2.145	1.963	1.525	1.736	1.418	-0.541	2.223	1.554	2.348	2.137	2.158	2.46	1.919	1.737	1.922	1.997	CRABP2

NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	CD3					
0.944	0.891	0.919	0.685	0.85	-0.189	0.987	0.723	1.058	1.449	1.758	1.277	1.209	0.901	1.175	1.211	CNT3
1.32	1.254	2.22	1.152	1.191	0.224	1.297	1.272	1.296	1.739	2.459	1.485	1.049	1.026	1.317	1.574	SR-A
1.805	1.057	1.266	1.393	0.526	-0.72	1.609	1.091	1.377	1.479	1.518	1.377	1.153	0.865	1.078	0.832	Hypothetical Protein
1.83	1.347	1.458	1.599	0.867	-0.473	1.898	1.702	1.634	1.715	1.613	1.767	1.736	1.339	1.75	1.393	Hypothetical Protein
1.609	1.192	0.865	1.263	0.48	-1.588	1.982	1.414	0.804	1.672	2.121	0.904	1.304	1.073	1.21	0.976	SCL2A5
0.983	0.732	0.758	0.749	0.615	-1.075	0.477	0.85	0.186	1.495	1.715	0.41	0.634	0.585	1.103	0.934	SULT1C1
2.414	2.21	2.335	2.377	1.987	-1.413	0.952	2.096	2.115	2.188	3.993	2.173	2.199	1.772	2.156	2.124	Chitinase-1
1.19	0.477	0.5	0.885	0.02	-1.009	0.658	1.019	0.971	1.007	1.283	0.994	0.965	0.525	0.922	0.75	RGSZ1
1.876	1.595	1.349	1.58	1.485	0.775	1.802	1.831	1.92	2.377	3.148	2.056	2.258	2.153	2.196	2.258	Cathepsin K
-0.627	-0.055	2.022	-0.861	-0.397	-0.368	2.105	-0.619	1.775	1.944	-0.043	1.863	1.01	0.874	1.184	0.815	EST
1.224	0.843	0.581	1.133	0.647	-1.866	0.803	1.142	1.08	1.195	1.358	1.086	1.023	0.943	1.126	1.204	Cyclophilin C
0.644	0.312	0.666	0.872	0.415	-0.442	0.38	1.119	1.171	1.038	1.882	1.386	1.142	1.104	0.915	1.024	EST
1.413	1.33	0.537	1.222	1.337	-0.219	0.603	1.108	1.876	2.093	1.574	1.739	1.684	1.466	1.537	1.754	Prostaglandin D2 Synthase
-1.122	0.531	1.476	-1.208	1.25	0.312	1.29	1.623	1.477	1.64	2.1	1.601	1.607	1.481	1.456	1.307	LILRA3
1.03	0.507	0.397	0.615	-0.073	-1.495	1.183	0.353	0.482	1.175	0.785	0.611	0.537	0.455	0.705	0.497	Hypothetical Protein
0.985	0.803	0.789	0.602	-0.153	-1.793	0.482	1.153	0.747	0.467	1.567	0.612	1.108	0.787	0.95	0.712	MRLC-2
0.922	0.375	0.53	0.794	-0.178	-1.473	0.282	0.712	0.805	0.971	1.119	0.765	0.667	0.643	0.777	0.53	TAZ
0.718	0.074	-0.38	-0.173	-0.256	-1.215	-0.51	0.321	0.735	0.512	0.771	0.72	0.675	-0.027	0.41	-0.009	HYA22
0.635	0.575	0.878	0.215	-0.347	-1.413	0.955	0.565	0.982	0.6	1.076	0.935	0.975	0.53	0.687	0.603	CD49C
0.532	-0.022	-0.31	0.389	-0.198	-3.001	0.8	0.315	0.621	0.454	0.536	0.538	0.479	0.377	0.445	0.539	Hypothetical Protein
0.504	0.308	-0.673	0.217	-0.862	-1.588	0.155	0.219	0.379	0.277	0.63	0.322	0.585	0.269	0.587	0.663	h-SmLIM
0.485	0.135	0.16	-0.48	-0.365	-0.396	0.574	0.764	0.424	0.795	0.999	0.435	-0.549	0.335	0.701	0.548	Neurofilament, Heavy Polypeptide
0.391	0.173	-1.386	-0.378	-0.269	-1.829	-0.096	0.52	0.434	0.067	0.042	0.475	0.147	-0.04	1.017	0.521	ROMK1
0.38	-0.005	-0.423	0.117	-0.488	-1.793	0.129	0.363	0.072	0.088	0.549	0.227	0.174	0.04	0.21	-0.444	HYA22
0.357	-0.088	-0.17	-0.212	-1.136	-1.323	0.889	0.158	0.011	0.214	0.234	-0.103	0.263	0.106	0.152	-0.41	Tropomyosin-2
0.347	0.525	1.319	0.015	0.579	-1.698	0.427	0.28	0.463	0.956	1.139	0.484	0.887	0.157	0.699	0.98	SCL7A8
0.065	0.427	0.178	-1.056	-0.083	0.191	0.465	-1.082	0.784	1.163	0.923	0.746	0.361	-0.098	0.137	0.27	Gelsolin
0.058	0.331	0.604	-0.426	0.316	-1.76	-0.123	-0.416	0.235	0.546	0.957	0.169	0.472	-0.209	0.293	0.642	SCL7A8
0.046	-0.214	0.609	-0.37	-1.285	-1.668	0.457	0.072	0.468	-0.231	-0.008	0.604	0.132	-0.359	0.4	0.119	FHL1
-0.001	-0.238	1.673	-0.241	-0.158	-1.829	-0.361	0.497	-0.117	0.104	1.697	-0.194	0.012	-0.203	-0.099	-0.025	MSR1
-0.053	-0.009	-1.712	-0.776	-1.554	0.271	-0.248	-0.013	0.478	0.462	0.349	0.43	0.305	-0.172	0.22	-0.25	Plakoglobin
-0.07	0.092	-1.511	-0.311	-0.691	-0.734	2.351	-0.6	-0.347	1.449	0.402	-0.468	0.323	0.548	0.109	-0.374	Tryptase
-0.412	-1.022	-1.403	-0.767	-1.285	-1.564	2.347	-1.097	-1.142	1.446	0.29	-1.066	0.361	0.521	-0.346	-1.015	Tryptase
-0.434	-0.242	0.026	-0.915	-0.774	-1.075	-0.288	0.465	0.209	-0.073	0.408	0.073	-0.015	-0.395	0.26	-0.155	VWF
-0.578	-1.022	-0.554	-1.119	-1.285	-1.588	-0.806	0.283	0.044	-0.221	0.449	-0.206	0.126	-0.303	-0.056	-0.21	Hypothetical Protein
-0.66	0.088	0.692	-1.767	0.047	0.07	-1.216	0.011	-0.055	0.553	0.867	0.301	0.212	-0.318	0.109	0.292	TRAP220

NIL			MDP			NIL			MDP			NIL	MDP	NIL	MDP	
HC1	HC2	HC3	HC1	HC2	HC3	CD1	CD2	CD3	CD1	CD2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
-0.724	-0.419	1.731	-0.3	-0.547	-0.95	1.629	-0.458	1.32	1.498	-0.818	1.511	0.744	0.609	0.881	0.376	EST
-0.823	-0.005	-0.926	-1.071	0.045	-1.122	-1.015	-1.388	0.192	0.643	0.514	0.156	-0.095	-0.403	-0.174	0.335	EMILIN
-1.169	-0.695	-0.035	-0.938	-1.505	-1.829	-1.605	-0.463	-0.222	0.102	0.158	-0.14	-0.208	-1.442	-0.85	-0.708	Myosin VI